Characterization of the Mitochondrial DNA Polymerase in *Plasmodium falciparum*

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To my family and friends – without the love and support you all have provided me over the years, I could not have completed my education with my sanity intact. For that I am eternally grateful! I love everyone with all of my heart.

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ABSTRACT

Characterization of the Mitochondrial DNA Polymerase in *Plasmodium falciparum*

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Several anti-malarials in clinical use and under development act on the mitochondrial electron transport chain (mtETC) of malaria parasites. The mtETC is an essential process requiring three proteins encoded by the mitochondrial DNA (mtDNA): cytochrome *c* oxidase subunits 1 and 3, and cytochrome *b*. The mitochondrion of *P. falciparum* relies on nuclearly encoded machinery for the replication, transcription and translation of the mtDNA. A candidate nuclear gene encoding mitochondrial DNA polymerase (mtDNAP) has been identified in *P. falciparum*. However, to our knowledge this gene has not been characterized. Due to the essentiality of the *P. falciparum* mtETC as demonstrated by previous studies, mtDNAP is assumed to be essential for parasite survival. Using single crossover homologous recombination, we have derived transgenic blood stage parasites with their endogenous mtDNAP locus tagged with TetR-DOZI aptamers. These transgenic parasites were used for conditional knockdown studies,

demonstrating a successful knockdown of mtDNAP. Surprisingly, this was not accompanied by any change in parasite growth. Further analysis of the mtDNA from knockdown parasites revealed a decreased amount of mtDNA, consistent with at least partial knockdown of the mtDNAP. This study demonstrates that *P. falciparum* parasites can survive with a significantly decreased mtDNA, allowing opportunity for future studies.

Chapter 1: Introduction to Malaria & the Mitochondria of *Plasmodium falciparum*

Malaria

Malaria continues to be a global burden that is increasingly difficult to treat due to the development of drug resistant parasites to all current treatments (*1*). In 2015, there were an estimated 429,000 deaths worldwide as a result of malaria, the majority of which were children under the age of five within Africa (*2*). The incidence of malaria deaths have decreased 21% between 2010 and 2015, along with a global decrease in mortality by 29% (*2*). This is primarily attributed to increased vector control in the form of insecticide-treated mosquito nets and indoor insecticide spraying to prevent transmission of malaria (*2*).

Current drug interventions involving artemisinin-based combination therapies (ACTs) for uncomplicated malaria have proven very effective against the most virulent of human malaria causing parasites, *Plasmodium falciparum* (*3, 4*). Although the burden of malaria continues to diminish, newly emerging drug resistant parasites against ACTs and other treatments requires a continued effort to find new drug interventions against malaria parasites. This effort is necessary to achieve global eradication of the disease. Recent studies have demonstrated that the mitochondrion of *P. falciparum* is a promising target for new anti-malarial drugs, due to its divergence from host mitochondria and the essential nature of the three proteins encoded by the parasite mitochondrial DNA (mtDNA), which will be discussed in detail (*5*).

Life Cycle of *Plasmodium falciparum*

Malaria in humans is caused by infection with apicomplexan parasites of the genus *Plasmodium*, which are transmitted by the bite of female *Anopheles* mosquitoes harboring the parasites, as male mosquitoes do not take blood meals (*6*). Severe malaria is most commonly caused by *P. falciparum*, but the disease may also arise from infection with *P. vivax* and *P. ovale*, which can uniquely remain dormant in the liver for up to a year, leading to multiple relapses of disease (*6*). In addition to these three species, human malaria is also caused by *P. malariae* and *P. knowlesi*. All of these parasites undergo a complicated life cycle, alternating between infection of the mosquito vector and the human host (**Figure 1.1**) (*6*).

Malaria infection by *P. falciparum* begins when an infected mosquito takes a blood meal from a human host, transmitting the disease by injecting sporozoites from the salivary glands during feeding. Upon entry into the blood stream, sporozoites travel to the liver, where liver stage schizonts develop over the course of approximately one week within hepatocytes. Mature schizonts rupture releasing merozoites, which infect erythrocytes, or red blood cells (RBCs). This initiates the asexual blood stage of the *P. falciparum* life cycle, which occurs cyclically over the course of 48 hours. Within the erythrocytes, merozoites develop into early trophozoites, or ring stage parasites, and then mature trophozoites differentiate into schizonts. As in the liver stage, the schizonts

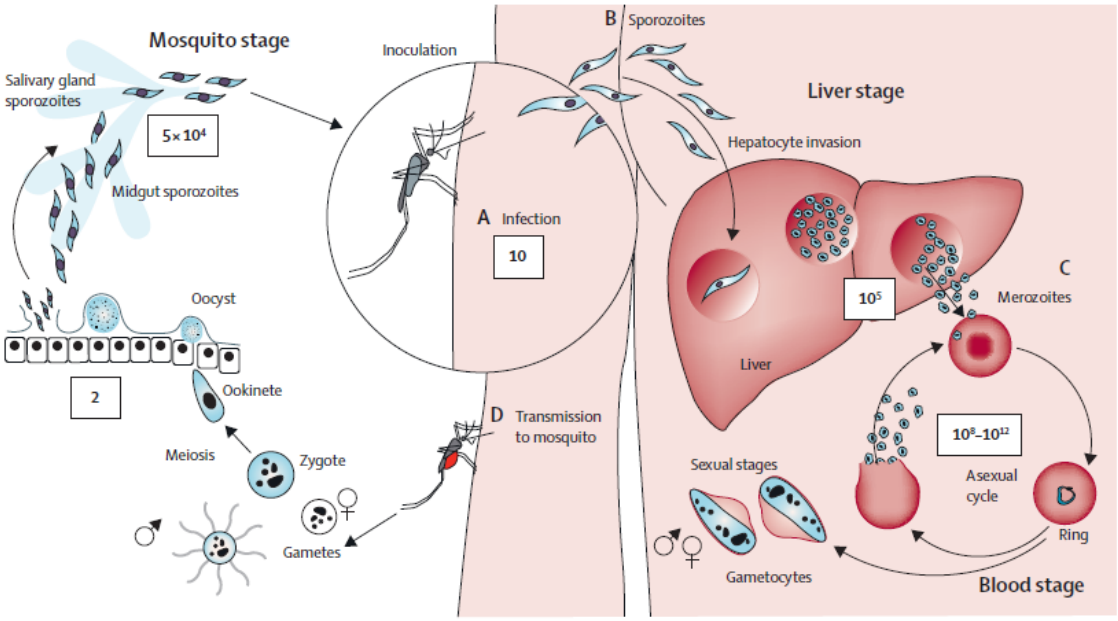

Figure 1.1: The life cycle of *Plasmodium falciparum*. The cycle begins with the bite of an infected mosquito, injecting the sporozoites into a human host. The sporozoites migrate to the liver to invade hepatocytes and further develop into schizonts over approximately one week, which burst and to release thousands of merozoites. These merozoites invade erythrocytes, beginning the asexual blood stage of infection. The merozoites will develop over 48 hours into early trophozoites (rings), mature trophozoites, and then schizonts, which will burst releasing merozoites for reinvasion of erythrocytes. Some trophozoites will develop into male and female gametocytes for sexual reproduction within the mosquito, if taken up during a blood meal. The gametocytes will form a zygote in the gut of the mosquito, which further develops into a motile ookinete that migrates to the salivary glands of the mosquito for oocyst development. The oocyst will burst, releasing sporozoites for reinfection should the mosquito bite a susceptible host. Republished with permission from (7).

rupture releasing 6 to 30 merozoites per schizont for reinvasion of erythrocytes, thus reinitiating the blood stage cycle (*7*).

A portion of invading merozoites develop into male and female gametocytes, which when taken up by a female *Anopheles* mosquito during a blood meal, allowing sexual reproduction of the parasites to begin within the gut of the mosquito. Zygotes develop into motile ookinetes, invade the midgut and form oocysts that rupture to release infectious sporozoites which migrate into the salivary glands. When an infected mosquito take a blood meal from a susceptible host, infection will occur and the life cycle will begin again (*8*).

Presentation of Disease

Clinically, malaria caused by *P. falciparum* is characterized into either uncomplicated or severe cases, with an incubation period of approximately two weeks (*9*). Early signs of uncomplicated malaria include an irregular fever, becoming more cyclic in pattern after one week, along with flu-like symptoms (*10*). Treatment for uncomplicated malaria typically includes ACTs, rapidly curing approximately 90% of cases (*7*). Without immediate treatment, more severe and potentially fatal symptoms can develop (*7*). Severe malaria consists of the same symptoms as uncomplicated malaria, and is classified as having one or more of the following complications including, severe anemia, cerebral malaria, respiratory failure and acute renal failure (*10*). The preferred treatment for severe malaria is

artesunate, a derivative of artemisinin, due to its high efficacy and low cost compared to other anti-malarial drugs (*7*).

The cyclic nature of the bursting of erythrocytes during the asexual blood stage causes anemia, as new erythrocytes are not replenished fast enough (*9*). The synchronous rupture of erythrocytes releases parasite toxins and antigens, which stimulate T cells to produce pro-inflammatory cytokines, such as IFN-γ and TNF-α, resulting in a corresponding cyclic fever and chills characteristic of malaria (*11*). Upon repeated exposure to parasitic infections, individuals can begin to exhibit a low level of immunity as a result of malaria-specific antibodies and T-cells, causing less severe symptoms and quicker clearance of parasites (*11, 12*). Alternatively, some individuals experience severe symptoms again due to cyclic cytokine production (*12*).

Malaria parasites also exhibit great antigenic variation as a means to subverting the immune response, allowing infection to persist for an extended time (*9*). Individuals with multiple exposures to parasitic infection are capable of developing natural immunity over a long period of time, likely producing multiple antibodies to a variety of epitopes presented by the parasites (*7*). This level of protection has yet to be reproduced by a vaccine (*7, 12*). Antigenic diversity is the primary cause of drug resistant parasites, contributing to the increasing difficulty of treating malaria (*12, 13*).

Drug Resistance

Unfortunately, resistance to artemisinin is an emerging problem throughout Southeast Asia, potentially undermining current strides in malaria treatment and prevention (*14*). Prior to artemisinin, chloroquine and sulfadozine-pyrimethamine were the primary treatments of choice, with resistance to these drugs developing throughout Southeast Asia, spreading to India and Africa shortly after (**Figure 1.2**) (*15*). Given this history of drug resistance, it is imperative that artemisinin resistance is closely monitored, which is now possible using the newly discovered kelch 13 mutation as a molecular marker in resistant parasites (*15*).

In order to fully understand the development of artemisinin resistance in *P. falciparum*, studies were conducted on lab isolates from Tanzania (*16*). Under artemisinin pressure eight mutations occurred across seven genes, with the most promising mutation located within chromosome 13, as this mutation has been frequently observed in other studies (*16-18*). Further research determined that there were many non-synonymous single nucleotide polymorphisms located in the kelch propeller domain (kelch13), with these mutations identified in resistant parasite isolates from Cambodia as well (*16*). Although it is not fully understood why the kelch13 mutations provide protection for the parasites, this mutation can now be used as a molecular marker to better monitor the spread and scale of artemisinin resistance (*15*).

Figure 1.2: Spread of chloroquine resistance from the Mekong region of Cambodia throughout Southeast Asia and Africa, with resistance also emerging in South America, from 1957 through 1990. Republished with permission from (*19*).

Anti-malarials, such as atovaquone and quinolones, act on the Q-cycle within complex III of the mitochondrial electron transport chain (mtETC) in malaria parasites and have been successful in treating the disease (*5*). Like artemisinin, high levels of resistance to atovaquone and similar drugs targeting the ubiquinol oxidation site of cytochrome *b* have quickly emerged (*20, 21*). To combat the growing resistance to atovaquone, addition of proguanil to drug treatments lowers the required dose of atovaquone for parasite clearance through a synergistic interaction, but once the resistance mutation at cytochrome b is developed by the parasites, the effect of both drugs are lost (*5*). The success of these drugs suggests the potential of additional drug targets against the parasite mitochondria, utilizing the evolutionary divergence from the host human mitochondria.

Evolution of the Mitochondria

The mitochondria of most eukaryotes are commonly referred to as the powerhouse of the cell and are integral parts of metabolism. Creation of the mitochondria occurred by an endosymbiotic event with alphaproteobacteria, thus forming eukaryotic cells (*22*). Within the phylum Apicomplexa, including *P. falciparum*, mitochondria are long organelles with tubular cristae, allowing for optimal surface area within the organelle for metabolic reactions (*23*). The main processes of the mitochondria are the tricarboxylic acid (TCA) cycle and mtETC.

Typically, the TCA cycle functions to oxidize acetyl-CoA through a series of reactions, producing CO_2 and some ATP molecules, as well as electrons to fuel oxidative phosphorylation within the mtETC, the primary source of ATP production (*24*). However, studies have demonstrated that blood stage *P. falciparum* parasites rely on ATP generation from the conversation of glucose to pyruvate during glycolysis, with the TCA cycle being non-essential for parasite survival (*25*). This discovery also brings into question the purpose of the mtETC within the parasite, to be discussed in the following section.

Although the mitochondria of eukaryotes are descendent of an alphaproteobacterium, the inherited genome of the mitochondria has evolved across alveolate species to be highly diverse, with *Cryptosporidium* species losing their mitochondrial genome completely (*5*). The Alveolate clade contains three major groups including ciliates, dinoflagellates, and apicomplexans, all with great differences in the evolution of their mitochondrial genome. Ciliates have the largest mtDNA of all the groups at approximately 47kb, containing 45 open reading frames (ORFs), 20 of which are unique to the ciliates (*26*). The dinoflagellates and apicomplexans have a much smaller mtDNA, each with only three ORFs, implying that the divergence of these groups occurred after significant loss of the mtDNA (*5*). Differences between dinoflagellates and apicomplexans are observed in the arrangement of mtDNA, with the former encoding on multiple DNA molecules and the latter demonstrating DNA molecules arranged in head-to-

tail tandemly repeated arrays (*27, 28*). The ORFs of both groups encode for the same three proteins, cytochrome *c* oxidase subunits I and III and cytochrome *b*, all required for the electron transport chain (*5*).

The Mitochondrial Electron Transport Chain

The mtETC is responsible for producing a transmembrane proton gradient across the inner membrane of the mitochondria through the reduction of oxygen to water (*24*). This proton gradient fuels oxidative phosphorylation to produce ATP from ATP synthase, also known as complex V, the primary source of ATP in eukaryotic cells (*24*). In addition to ATP synthase, eukaryotes have four complexes within the mitochondrial inner membrane that function to transfer electrons to a higher energy state and pump protons against the gradient of the inner membrane (*29*). The complexes consist of NADH-ubiquinone oxidoreductase (complex I), succinate-ubiquinone oxidoreductase (complex II), ubiquinol-cytochrome *c* oxidoreductase (complex III), and cytochrome *c* oxidase (complex IV) (*5*). Complexes I, III, and IV function to pump protons against the gradient of the inner membrane into the inner membrane space, generating a protonmotive force, while complex II introduces electrons derived from succinate but does not function as a proton pump (*30*). There are also two additional proteins, ubiquinone (Coenzyme Q) and cytochrome *c*, which shuttle electrons between the complexes (*30*).

As previously noted, *P. falciparum* in the asexual stages generates ATP through glycolysis due to the high availability of glucose (*31*). Although these blood stage parasites do not use the mtETC to produce ATP, it is still essential for other processes, including maintenance of the membrane potential within the mitochondria and pyrimidine biosynthesis (*29*). The mtETC of *P. falciparum* is similar to higher eukaryotes, and is generally more simplified with the loss of complex I, as seen in **Figure 1.3** (*29, 31*). Electron transfer between complexes II-IV occurs as in higher eukaryotes but with less activity, the only exception being the loss of some subunits within the complexes, again demonstrating a simpler system (*29*). Complex III, or cytochrome bc_1 complex, of the *P. falciparum* mtETC is of particular interest, due to its susceptibility to inhibitors such as atovaquone, which bind the ubiquinol oxidation site (Q_o) of the complex (*32, 33*). By binding and blocking the Q_o site, electrons cannot be transferred and ubiquinone is not reoxidized to ubiquinol (*33*). Atovaquone resistance is directly related to a mutation in cytochrome *b* at position 268, a tyrosine residue, altering the Q_o site and preventing atovaquone, as well as other inhibitors, from functioning (*33*).

Five mitochondrial dehydrogenases within the mtETC of *P. falciparum* contribute to the Q cycle by reducing coenzyme Q (CoQ) and feeding electrons into the mtETC (*31*). Specifically the dihydroorotate dehydrogenase (DHODH) has an additional role in pyrimidine biosynthesis, an essential pathway for survival as parasites cannot salvage pyrimidines (*5*). DHODH catalyzes the conversion of

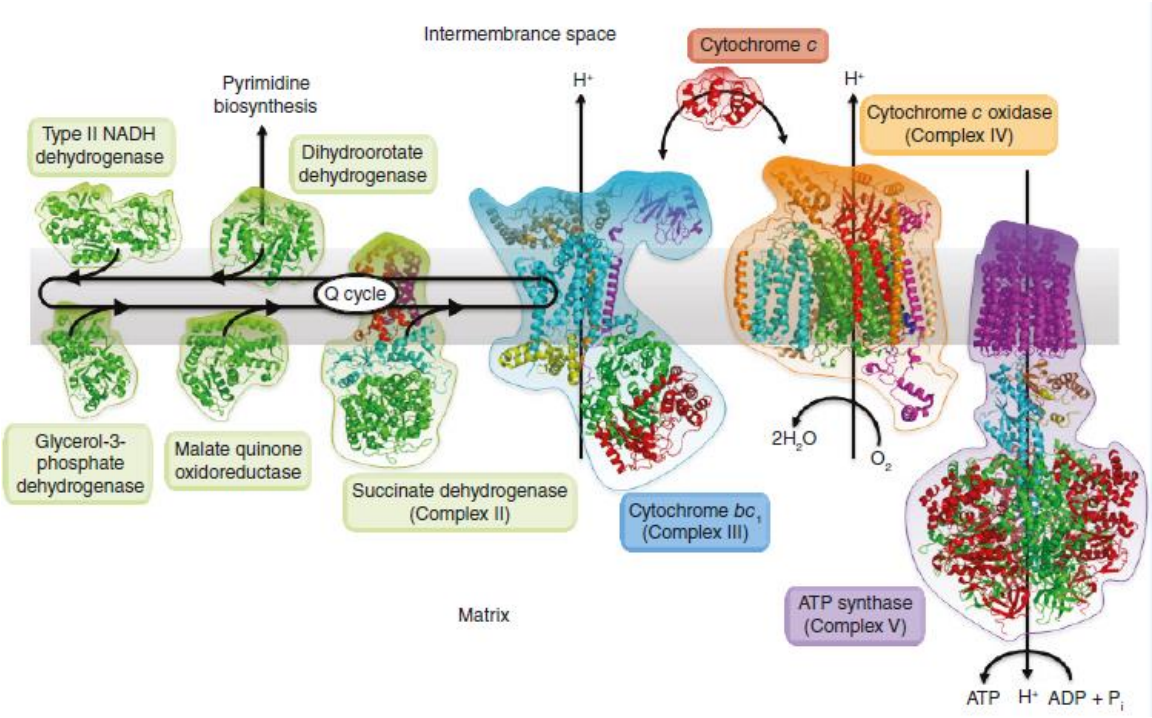

Figure 1.3: The mitochondrial electron transport chain of *P. falciparum*, including the five dehydrogenases of the Q cycle, complexes II-V, and cytochrome *c*. Republished with permission from (*31*).

dihydroorotate to orotate, the only redox reaction in the pyrimidine biosynthesis pathway (*34*). A study by Painter et al. (*35*) expressed a class 1 yeast DHODH (yDHODH) in *P. falciparum* blood stage parasites, producing parasites resistant to complex III inhibitors, including atovaquone. These transgenic parasites functioned completely independent of the mtETC, as yDHODH uses fumarate as an electron acceptor instead of CoQ, allowing pyrimidine biosynthesis to occur without reliance on the Q cycle. Interestingly, transgenic yDHODH expressing parasites were susceptible to proguanil in combination with complex III inhibitors, indicating that the drug targets a function that is only essential without the mtETC. Further study confirmed that proguanil in the presence of atovaquone disrupts the membrane potential of the mitochondria, leading to collapse of the membrane and parasite death. This work demonstrates the essential nature of the mtETC, and questions if mtDNA responsible for encoding three of the required proteins for this process can be manipulated as well.

The Mitochondrial DNA

The mtDNA of *P. falciparum* is a total of 6 kb in length, the smallest mtDNA known within eukaryotes (*36*). The mtDNA encodes only three genes, Cox1, Cox3 and Cytb, which produce the proteins cytochrome *c* oxidase subunits 1 and 3, and cytochrome *b*, as stated previously, and the ribosomal RNA required to produce ribosomes for translation of these proteins (**Figure 1.4**) (*5, 37*). The

transcription and translation of mtDNA requires nuclearly encoded machinery, transfer RNAs, and polymerases to be imported into the mitochondria after translation in the cytosol of the parasites (*29*). All other proteins required for the mitochondria are also nuclear encoded and transported to the mitochondria after translation (*29*).

There are approximately 30 copies of the mtDNA arranged in head-to-tail tandem arrays, with replication occurring in a unique mechanism not commonly seen in eukaryotes (*38*). Initiation of mtDNA replication coincides with the start of nuclear replication at the late trophozoite stage during the asexual blood cycle (*39*). Studies have confirmed that the replication of mtDNA occurs by a rolling circle mechanism, with multiple single stranded copies of the DNA produced in a head to tail arrangement called a concatemer (*39*). This method of replication is thought to arise due to the complex aggregates formed from the intramolecular recombinational activity of linear concatemers within the mitochondria (*39*). To further investigate the requirement of replicating these three genes, the mitochondrial RNA polymerase (mtRNAP) of *P. falciparum* has been studied as a possible anti-malarial drug target.

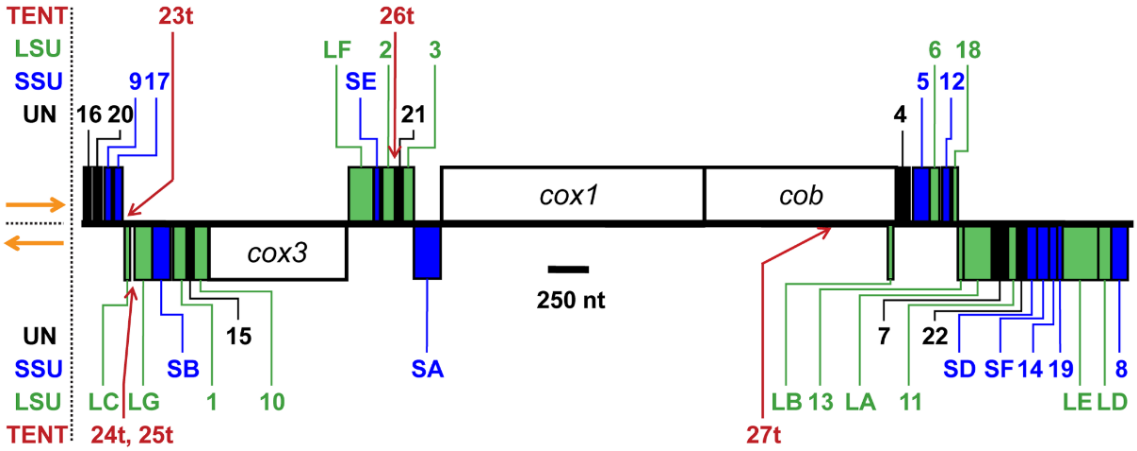

Figure 1.4: The mitochondrial genome of *P. falciparum*. The map depicts the 6kb mitochondrial DNA, with genes above the line transcribed left to right and genes below the line transcribed right to left. This sequence is tandemly repeated to form the entire genome. The genes *cox1*, *cox3* and *cob* produce the proteins cytochrome *c* oxidase subunits 1 and 3, and cytochrome *b*, respectively. Protein coding regions are indicated by white boxes, with small mitochondrial rDNA sequences for the large subunit (LSU) rRNAs shown in blue (rRNA fragments SA-SF) and the small subunit (SSU) rRNAs shown in green (rRNA fragments LA-LG). Two tentative rRNAs (TENT) are shown in red, with the untranslated regions (UN) shown in black. Republished with permission from (*37*).

The Mitochondrial RNA Polymerase

The mitochondrial RNA polymerase (mtRNAP) (PF3D7_1125300) of *P. falciparum* is a nuclearly encoded protein required for transcription of genes replicated from the mtDNA (*40, 41*). Another proposed purpose of the mtRNAP is for generation of RNA primers to initiate replication of the mtDNA (*40*). A recent study by Ke et al. (*41*) demonstrated that mitochondrial RNA polymerase (mtRNAP) is essential in asexual blood stage parasites since gene knockout attempts were unsuccessful. The goal of the study was to produce parasites lacking the mtDNA for further investigation of the functions of the mitochondria in parasites. Knockouts were conducted in yDHODH-expressing parasites independent of the mtETC and should not require the mtRNAP, as proteins encoded from the mtDNA would no longer be necessary; however, the mtRNAP gene could not be knocked out. Due to the essential nature of mtRNAP in *P. falciparum*, another potential drug target could be the mitochondrial DNA polymerase (mtDNAP) (PF3D7_0625300), an enzyme that has yet to be well characterized.

The Mitochondrial DNA Polymerase

The mtDNAP in *P. falciparum* is a proposed γ-like polymerase with some homology to eukaryotic DNA polymerase γ, as both function to replicate the mitochondrial DNA (*42*). A study by Petmitr et al. (*42*) previously investigated the

enzymatic activity of the mtDNAP by partially purifying it from isolated blood stage *P. falciparum* mitochondria using fast protein liquid chromatography (FPLC). The polymerase was characterized to be mitochondrial based on resistance and susceptibility to known DNA polymerase inhibitors, as different DNA polymerase types have varying susceptibilities (*43*). DNA polymerases in eukaryotes can be classified into two categories, aphidicolin-sensitive and aphidicolin-resistant, the latter of which only includes DNA polymerase β and γ (*43*). Of those two DNA polymerases, β is resistant to *N*-ethylmaleimide while γ is susceptible, and this difference was utilized to classify the purified polymerase. The purified enzyme showed resistance to aphidicolin and susceptibility to *N*-ethylmaleimide, both of which have been observed in eukaryotic DNA polymerase γ, confirming that it is a γ-like DNA polymerase. The polymerase activity of the purified mtDNAP was analyzed using activated calf thymus DNA as a template to quantify enzyme activity, and polymerase activity was observed in the presence of aphidicolin. This study characterized the enzymatic profile of the mtDNAP, but requires further investigation to confirm that the polymerase primarily functions in the mitochondria.

Specific Aims

Until recently, there was little to no information on the genomic sequence of *P. falciparum* DNA polymerases of any type, limiting further research. Since the

study by Petmitr et al., the *P. falciparum* genome sequencing project determined the putative genomic sequence of mtDNAP, along with many other genes, and it can now be utilized to further study and manipulate the enzyme (*44*). With confirmation of the presence of a DNA polymerase in the *P. falciparum* mitochondria by Petmitr et al., studies can be completed on the genomic level in order to determine the essentiality of the protein. Based on the study by Ke et al., it is likely that the mtDNAP will exhibit similar properties as the mtRNAP, since the mtRNAP is essential for transcription of messenger RNA (mRNA) from replicated mtDNA by the mtDNAP. The mRNA is most likely necessary for production of proteins required outside of the mitochondria, because otherwise the mtRNAP would have been able to be disrupted. Given this information, we hypothesized that the mitochondrial DNA polymerase is essential for parasite survival due to the requirement of the mitochondrial DNA polymerase for replication of mtDNA in *P. falciparum* parasites. The specific aim of this thesis is to determine whether mitochondrial DNA polymerase is essential for the survival of blood stage *P. falciparum* by using a conditional knockdown system and growth assay analysis.

Chapter 2: Materials & Methods

Plasmid Construction for Knockdown of mtDNAP

The pMG75/TetR-DOZI plasmid from Ganesan et al. (*45*) was modified for knockdown of *P. falciparum* mtDNAP. The PfATP4 gene was removed from the pMG75 plasmid by restriction digest using specific restriction endonucleases (New England Biolabs) for the AflII (5') and BstEII (3') sites flanking the insert. A 1031 bp homology region at the 3' end of the mtDNAP (PF3D7_0625300) gene, immediately before the stop codon, was isolated by polymerase chain reaction (PCR) from wild type (WT) D10 parasite genomic DNA (gDNA) using gene specific primers listed in **Table 2.1**. Primers were designed with the same restriction sites used to remove PfATP4 from the plasmid. The mtDNAP insert was purified and digested with the same enzymes used on the plasmid and cloned into the linearized pMG75 plasmid, producing the final construct (**Figure 2.1**). The construct was transformed into competent cells, with kanamycin resistance for positive selection, and colonies were screened by PCR to confirm transformation of the full vector construct. Positive colonies with the full construct were verified by sequencing (Genewiz) and restriction digest. A single positive clone was grown in bacterial culture and a maxi prep kit (Qiagen) was used to isolate and purify the plasmid DNA. The pMG75/mtDNAP vector DNA was precipitated into sterile 50µg aliquots for parasite transfection. Blasticidin (BSD) resistance was used as the selectable marker for transgenic parasites in culture, with presence of anhydrotetracycline (aTc) to prevent premature knockdown of mtDNAP.

Plasmid Construction for Knockout of mtDNAP

The pUF-1 plasmid with the mtDNAP 5' and 3' homology regions was made previously by Suresh Ganesan and Hangjun Ke (**Figure 2.2**). In brief, the 5' homology region (5'HR) was cloned into the pUF-1 plasmid using specific primers (**Table 2.1**) with the NcoI and EcoRI restriction sites. The 3' homology region (3'HR) was cloned into the plasmid using specific primers (**Table 2.1**) with the SacII and SpeI restriction sites. Guide RNAs (gRNAs) between the 5'HR and the 3'HR were designed using the Eukaryotic Pathogen CRISPR guide RNA Design Tool (http://grna.ctegd.uga.edu/) developed by the University of Georgia to find specific PAM sites within the sequence (**Table 2.1**). These guides were ligated into the Cas2A plasmid by infusion. Cloning and preparation for transfection of the pUF-1 construct and Cas2A constructs occurred as previously stated, with ampicillin resistance used for positive selection. The plasmid contains yDHODH to generate DSM1 resistance as the selectable marker for transgenic parasites in culture.

Cloning Region	Primer Name	Sequence
mtDNAP forward insert (pMG75)	DNAPolI_F	ATCTTAAGCAACTAATAATGTATCAACCAATAATG
mtDNAP reverse insert (pMG75)	DNAPolI_R	TAGGTAACCATTGAAGACTCCTTGTAGACTCC
mtDNAP 5'HR forward (pUF-1)	5pDNApolsen_F	CTCCATGGAAGATGCAGATGAACATACAGATC
mtDNAP 5'HR reverse (pUF-1)	5pDNApolasen_R	GTGAATTCGATAATCTTCCTGTTGATGCTCC
mtDNAP 3'HR forward (pUF-1)	3pDNApolsen_F	CAACTAGTGAAAGTGAACATGATGCTACC
mtDNAP 3'HR reverse (pUF-1)	3pDNApolasen_R	TACCGCGGTCGTGTATACTTTAATTTGCATCCTC
mtDNAP gRNA 1 forward (pUF-1)	DNApl_gRNA1_F	CATATTAAGTATATAATATTCACCTTCGATTATAAACAAAGTTTTAGAGCTAGAAATAGC
mtDNAP gRNA 1 reverse (pUF-1)	DNApl_gRNA1_R	GCTATTTCTAGCTCTAAAACTTTGTTTATAATCGAAGGTGAATATTATATACTTAATATG
mtDNAP gRNA 2 forward (pUF-1)	DNApl_gRNA2_F	CATATTAAGTATATAATATTTTATATGGACAAACTGAAAAGTTTTAGAGCTAGAAATAGC
mtDNAP gRNA 2 reverse (pUF-1)	DNApl_gRNA2_R	GCTATTTCTAGCTCTAAAACTTTTCAGTTTGTCCATATAAAATATTATATACTTAATATG
mtDNAP gRNA 3 forward (pUF-1)	DNApl_gRNA3_F	CATATTAAGTATATAATATTTCTGCTGCACATCCTTGTATGTTTTAGAGCTAGAAATAGC
mtDNAP gRNA 3 reverse (pUF-1)	DNApl_gRNA3_R	GCTATTTCTAGCTCTAAAACATACAAGGATGTGCAGCAGAAATATTATATACTTAATATG

Table 2.1: Primer sequences for vector cloning. Primers used for cloning of the mtDNAP homology regions into the pMG75 plasmid and pUF-1 plasmid, and for cloning the guide RNAs into the Cas2A plasmid.

Cloning Region	Primer Name	Sequence
mtDNAP forward integration check	mtDNAP_Intg_F	AATCAAATTGGAGCATCAACA
mtDNAP reverse integration check	DNApl_2Xchk_R	CCTTCATTTATCATCATCATC
Aptamer reverse check	3'_TetR_Check	ATATTTCATGTCTCAGTAAAGTCTTTCAATAC
mtDNAP reverse 3'UTR flanking	mtDNAP3f_R	ATGACGTCGAAAAGTATTAATGATGATCTG

Table 2.2: Primers for PCR of pMG75 transgenic parasites. Primers used to check for vector integration, wild type parasites, and aptamer length, with the same forward primer for each reaction.

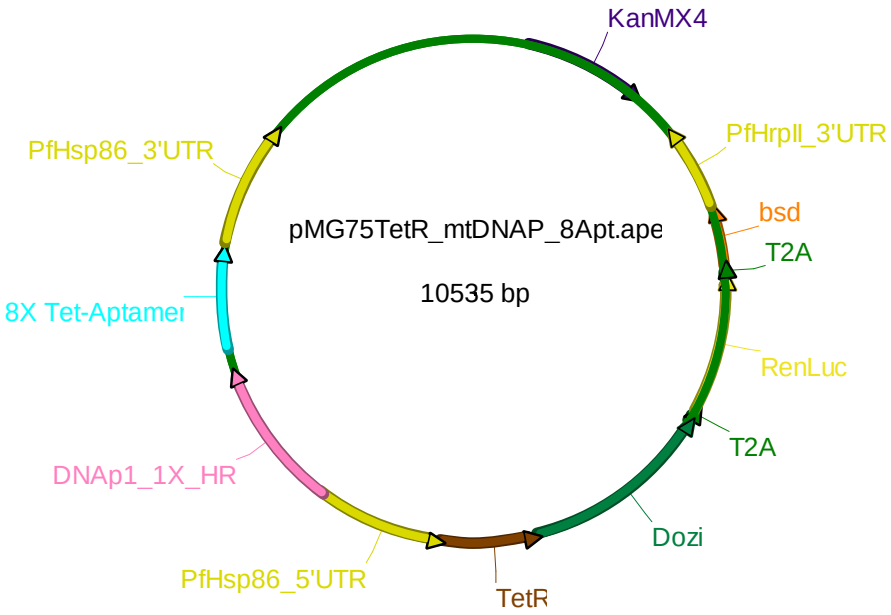

Figure 2.1: pMG75/TetR mtDNAP vector construct for single crossover recombination of plasmid into the 3'UTR after the mtDNAP gene.

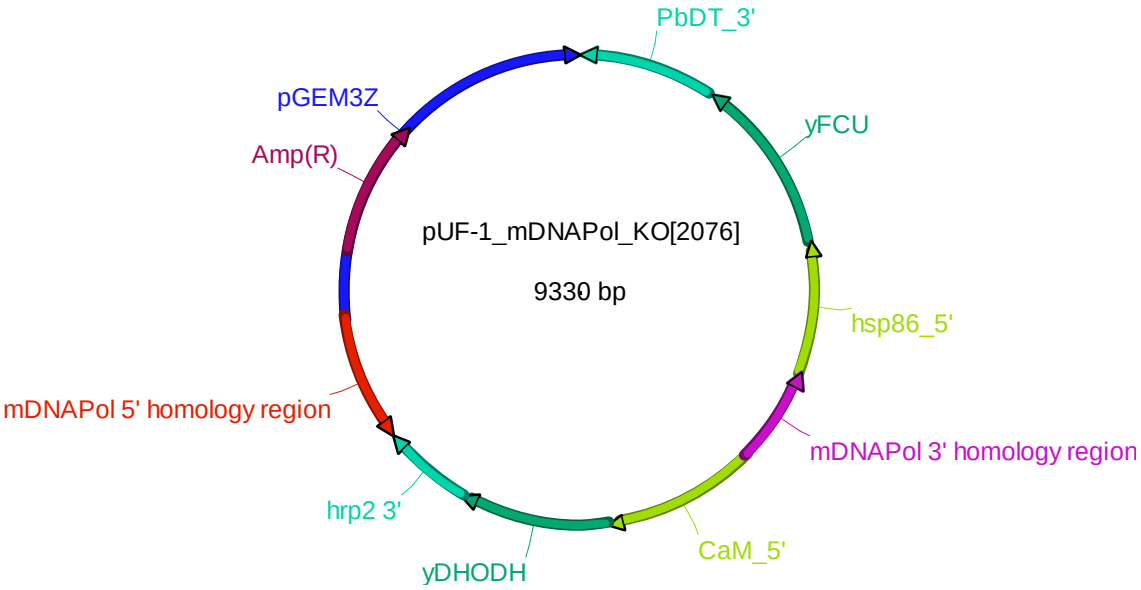

Figure 2.2: pUF-1 mtDNAP vector construct for double crossover recombination of plasmid at the 3' end of the mtDNAP gene after cutting by Cas9.

Parasite Culture & Transfection

The D10 strain of *Plasmodium falciparum* cultured in human O+ erythrocytes at 5% hematocrit in RPMI 1640 media was the primary wild type strain used for transfections, with some transfections in NF54. RMPI 1640 medium was supplemented with 10mg/L 3H-hypoxanthine (Sigma), 15mM HEPES (Hyclone), $NAHCO_3$ (Cellgro), 0.5% Albumax II (InVitrogen), and 50µg/mL gentamycin (Cellgro). Parasites were incubated at 37˚C, with a gas mixture of 6% O_2, 5% CO_2 and 89% N_2. Ring stage parasites were transfected by electroporation at approximately 5-7% parasitemia using 0.2cm cuvettes, with 250µL parasitized erythrocytes and 50µg of plasmid DNA in cytomix solution. Electroporation occurred at 0.31Kv and 960µFD, and parasites were transferred to a T25 vented flask with an additional 0.2mL of 50% RBCs and 9mL RPMI media. Drug selection media was added two days after transfection and parasites were maintained until a stable population recovered from electroporation.

Drug Cycling to Generate Integrated Transgenic Parasites

Once parasites recovered from transfection, integration of pMG75/TetR vector DNA into the parasite genome was confirmed by PCR analysis with flanking primers listed in **Table 2.2**. Presence of wild type resistant parasites and length of aptamer was also confirmed using specific primers (**Table 2.2**). Parasites were

isolated from red blood cells using saponin lysis (0.01% final concentration) and DNA was purified using the QIAamp DNA Blood Mini kit (Qiagen). Parasites without integration were drug cycled by maintaining BSD drug pressure for three weeks, followed by removal of BSD from the RPMI media for four weeks. Drug pressure is again added for an additional week, and DNA was collected from parasites to check for integration by PCR analysis.

Parasite Cloning by Limiting Dilution

Mixed parasite culture of transgenic parasites and resistant wild type parasites were cloned by limiting dilution as described previously by Ke et al. (*25*). Briefly, the culture was diluted to a 1:10 stock, and the erythrocyte cell density was determined using a hemocytometer. Parasitized erythrocytes per mL was determined by multiplying the cell density by the parasitemia. The 1:10 stock was diluted to final concentrations of 2.5 and 0.5 parasitized RBCs per mL, and plated into separate 96 well plates to provide an average of 0.5 and 0.1 parasitized RBC per well. Plates were maintained with regular feeding and a 1:2 split at day 10 and day 17. Once a parasite population was established, parasite clones were expanded to larger cultures and screened by PCR for transgenic and wild type parasites. Pure transgenic parasite clones were also checked for length of aptamer.

Immunofluorescence Assay

Transgenic parasites with Myc-tagged mtDNAP from the pMG75 plasmid are maintained in RPMI media at 5% hematocrit containing BSD and aTc. Parasites were prepared for immunofluorescent analysis as previously described by Tonkin et al. (*46*). Briefly, at 5-7% parasitemia, a 1mL sample was collected and treated with 60nM MitotrackerRed for mitochondrial staining. Cells were fixed with 4% formaldehyde and 0.0075% glutaraldehyde overnight at 4˚C, then permeabilized with 0.1% TritonX100 in PBS for 10 minutes, followed by 3X PBS washes. Cells were treated with 0.1mg/mL sodium borohydride in PBS for 10 minutes and then blocked with 5% BSA overnight at 4˚C. Blocked cells were treated with rabbit polyclonal Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then anti-rabbit AlexaFluor488 fluorescent antibody (Molecular Probes, Eugene, Oregon, USA), both at a 1:300 dilution in 1% BSA in PBS overnight at 4˚C, with 3X PBS washes between antibodies. Slides are prepared by treating stained samples with antifade and fluoromount for imaging on an Olympus BX60 epi-fluorescence microscope system.

Knockdown Assay

Ring stage D10 transgenic parasites were tightly synchronized using alanine and grown to 7-10% parasitemia. At the trophozoite stage, parasites were washed

3X with RPMI media and split 1:10 into fresh blood to remove excess aTc from the culture. Cultures were established in T75 flasks with 3mL total blood and were maintained RPMI/BSD media with or without aTc for 20 days, or 10 cycles. Every 24 hours, a smear slide was prepared and 50µL of parasitized RBCs (25µL packed) was collected for flow cytometry analysis from each flask. Every 48 hours, parasites were isolated by saponin lysis (0.01% final concentration) with ¼ of the flask collected for PCR analysis and ½ of the flask for Western blot analysis. Parasite pellets collected from the saponin lysis were stored at -20˚C until prepared for analysis. The remaining ¼ of the flasks were replenished with fresh blood to a final volume of 3mL RBCs.

Western Blot Analysis to Confirm Knockdown

Parasite pellets collected during the knockdown assay were resuspended to 5-10X volume of the pellet using 50mM Tris-HCl pH 6.8 and 2% SDS buffer. Samples were prepared using 20µL of the resuspended pellet, 8µL of 4X SDS loading buffer and 3µL of 5% β-mercaptoethanol. The parasite lysates were heated for 10 minutes at 85˚C and then centrifuged for 5 minutes at 14,000 RPMs. The samples were loaded onto a 4-15% gradient pre-cast SDS-PAGE gel (Bio-Rad Laboratories, USA) at 100V for 1.5 hours and transferred onto a polyvinylidene difluoride membrane activated with methanol at 100V for 1 hour. Membranes

were blocked in 10% non-fat milk in TBS-0.1% Tween for one hour. The membrane was probed with a 1:8000 dilution of rabbit polyclonal Myc antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 1% non-fat milk in TBS-0.1% Tween overnight at 4˚C. The membrane was washed 3X in TBS-0.1% Tween and probed with a 1:8000 dilution of goat anti-rabbit-IgG HRP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 1% non-fat milk in TBS-0.1% Tween overnight at 4˚C. The membrane was washed 3X in TBS-0.1% Tween and developed by autoradiography using the SuperSignal West Femto Chemiluminescent Maximum Sensitivity substrate (Thermo Scientific, Rockfield, IL, USA) at a 1:2 dilution in PBS for 5 minutes. The membrane was washed as above and then probed with HRP conjugated rabbit polyclonal *Plasmodium* Aldolase antibody (Abcam, Cambridge, MA, USA) at a 1:10,000 dilution as a loading control. The membrane was washed and developed by autoradiography using the SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific, Rockfield, IL, USA) for 5 minutes.

Growth Assay by Flow Cytometry

Parasite samples collected for flow cytometry were washed 3X in PBS and fixed in 4% formaldehyde and 0.0075% glutaraldehyde overnight at 4˚C. Samples were stored at 4˚C until prepared for staining. Fixed samples were washed 3X with

PBS and stained nucleic acid content with 1mM SYBR Green (Thermo Scientific, Rockfield, IL, USA) for 90 minutes while rotating. The stain was removed by again washing 3X with PBS. Stained samples were analyzed using the Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) on the FL1 channel for SYBR Green signal. Parasites were gated using unstained and stained RBCs as controls, with a signal intensity above approximately 10^5 fluorescent units indicating parasitized RBCs.

Quantitative PCR Analysis of mtDNA

Genomic DNA (gDNA) was purified from the parasite pellets collected during the knockdown assay using the QIAamp DNA Blood Mini kit (Qiagen). Total volume for each PCR reactions was 20µL with the following conditions: 10µL SYBR Green (Applied Biosystems, Warrington, UK), 1µL forward primer (10µM), 1µL reverse primer (10µM), 50ng gDNA based on sample concentration and H_2O up to 20µL. Primers used for quantitative PCR (qPCR) are listed in **Table 2.3**, with cytochrome *b* used to determine the presence of mtDNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used as a control gene. PCR reactions were plated in triplicate, with D10 WT gDNA and samples without gDNA used as controls. . To determine the fold change of the amount of mtDNA, the average for each sample was determined from the technical replicates. The D10 Ct mean was then subtracted from the Ct means of the samples for each gene, to determine the

ΔCt value for each sample. The GAPDH ΔCt for each sample was subtracted from the corresponding cytrochrome *b* ΔCt, to give the ΔΔCt for each sample. Then, the following formula was used to calculate the final fold change: $(-\Delta\Delta Ct)^2$. The fold changes were normalized to samples with aTc for each time point and plotted accordingly.

Growth Inhibition Assays

Growth inhibition assays were conducted as described in Desjardin et al. (*47*). Briefly, the parasite cultures were plated in 96 well plates at approximately 1.0% parasitemia in 3% hematocrit. Serial dilutions of specific drugs and inhibitors were added in the presence or absence of aTc and then pulsed with 0.5µCi of ^{3}H-hypoxanthine for 24 hours. The plates were frozen at -80˚C for 24 hours, then thawed to 37˚C to lyse the parasites and harvested onto EasyTabTM-C Self-aligning glass fiber filters (Packard, Meridian, CT, USA), followed by multiple washes. The filters are dried and placed into an OmnifilterTM 96-well plate filter case (Packard, Meridian, CT, USA). To measure the beta-radiation of ^{3}H-hypoxanthine incorporation, 30µL OmniScint™ (Packard, Meridian, CT, USA) scintillation fluid is added to the wells. A TopCount™ radiation counter is used measure cell proliferation by the counts per million (cpm) of radioactivity incorporation into the nucleic acids. The cpm is used to determine the percent growth, and then plotted to determine the best fit curve and the IC_{50} of each drug.

Cloning Region	Primer Name	Sequence
Cytochrome *b* forward	Cytochromeb_F	AGCAAGTCGATATACACCAGATG
Cytochrome *b* reverse	Cytochromeb_R	ACCTGTTGCGTGCATGTATC
GAPDH forward	rGAPDHPfF	TCCTTGGGGAAAATGCCAAGT
GAPDH reverse	rGAPDHPfR	TGGGGTGTCATCCTTTGGTG

Table 2.3: Primers for qPCR analysis. Cytochrome *b* primers generate a product of 106bp used to quantify the amount of mtDNA. GAPDH primers generate a product of 136bp used as a housekeeping gene control.

Chapter 3: Characterization of the Mitochondrial DNA Polymerase of *Plasmodium falciparum*

Conserved Domain Analysis of Mitochondrial DNA Polymerases

The mitochondrial DNA polymerase of *Plasmodium falciparum* is currently a putative gene, with no previous genomic studies completed. The function of this protein has been proposed based on conserved domain regions within the protein sequence to known polymerases. Localization of this protein to the mitochondrial is indicated by a mitochondrial targeting sequence encoded at the beginning of the predicted protein. Utilizing this targeting sequence is a strategy that has been used to identify multiple nuclear genes that encode proteins which localize to the mitochondria. Given this information, further investigation of the conserved domains within the mtDNAP of *P. falciparum* (*Pf*mtDNAP) can be completed using available databases.

In general, the conserved catalytic domain of the *Pf*mtDNAP falls in the DNA polymerase A family as shown in **Figure 3.1A**, one of six families of polymerases classified primarily by the family of the organism and the overall function of the polymerase (*48*). The polymerase A family includes polymerases with homology most similar to *Escherichia coli* polymerase I, and function to fill DNA gaps during DNA repair, recombination and replication (*49*). The complete family with specific sub-families are listed in **Figure 3.1D**. For reference, the mitochondrial polymerases of *Saccharomyces cerevisiae* and *Homo sapiens* were also analyzed (**Figure 3.1B-C**), with the latter offering particular interest as the host organism. Both organisms demonstrated conserved catalytic domains within

the DNA polymerase A family, and as expected fell into the more specific gamma sub-family. The gamma sub-family consists of polymerases responsible for DNA replication and repair within the mitochondria (*49*).

The *Pf*mtDNAP surprisingly lacked homology with the gamma sub-family, and fell into the more general category of polymerase family A. To further investigate the significance of this homology, the alignment of *Pf*mtDNAP with DNA polymerase family A (pfam00476) was generated, shown in **Figure 3.2A**. The low e-value of 6.12e-61 indicates significant conserved domain homology between *Pf*mtDNAP and family A based on the protein sequence, however some divergence is observed between specific amino acid residues (**Figure 3.2A**). The mtDNAP of *H. sapiens* demonstrated nearly perfect alignment with the gamma sub-family shown in **Figure 3.2B**, confirming the conserved domain function in mitochondrial DNA replication. The *Pf*mtDNAP was then aligned with the catalytic subunit of the *H. sapiens* mtDNAP to confirm divergence between pathogen and host, with the full alignment listed in **Appendix III**. This divergence is critical for potential drug development, should the *Pf*mtDNAP prove to be an anti-malarial drug target, to eliminate unwanted drug interactions with the host.

Figure 3.1: Conserved domains of multiple mitochondrial DNA polymerases. The mtDNAP of *H. sapiens* (**A**) and *S. cerevisiae* (**B**) show homology with the DNA polymerase A superfamily, specifically with the DNA polymerase gamma subfamily. The mtDNAP of *P. falciparum* (**C**) shows homology with the DNA polymerase A family. **D.** The sub-family hierarchy of the DNA polymerase A family, with the DNA polymerase gamma subfamily primarily involved in mitochondrial DNA replication. Conserved domains and images were generated by the NCBI Conserved Domain Database (*49*).

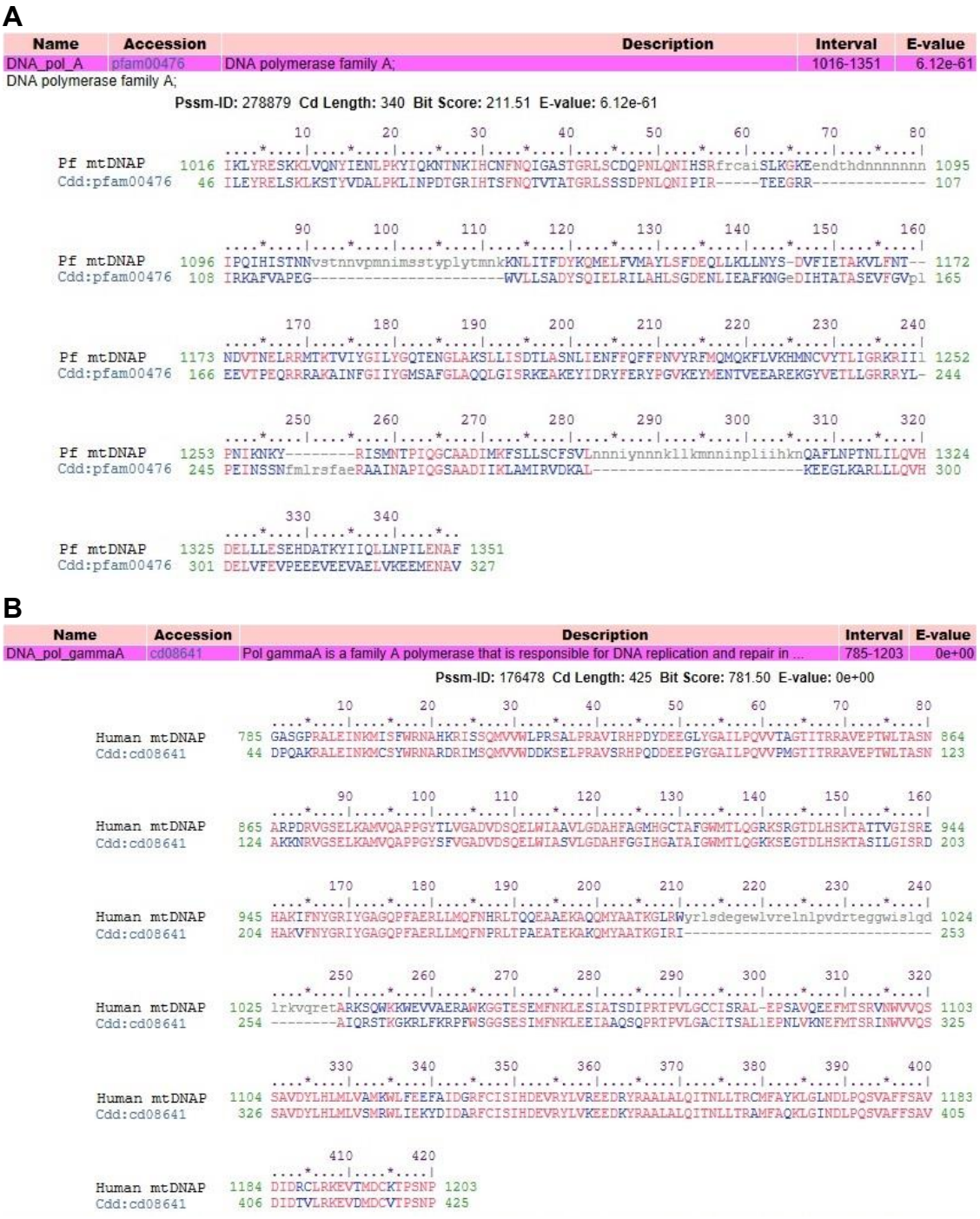

Figure 3.2: Protein alignments of mitochondrial DNA polymerases. **A.** *P. falciparum* mtDNAP (UniProtKB - C6KT89) is more closely related to DNA polymerase Family A. **B.** *H. sapiens* mtDNAP (UniProtKB - P54098) is more closely related to the DNA polymerase gamma subfamily. Alignments generated by NCBI Conserved Domain Database (*49*).

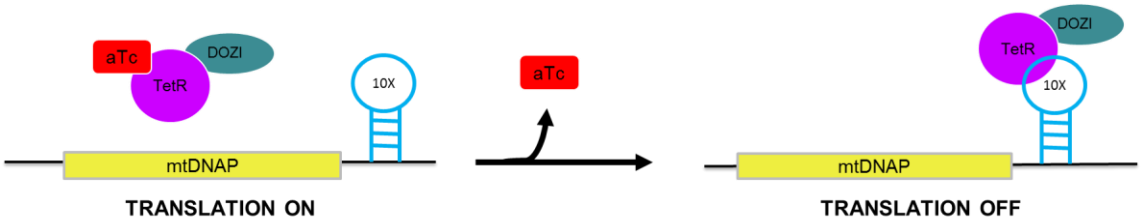

Figure 3.3: Conditional knockdown mechanism. The aptamers are encoded within the 3'-UTR of the mtDNAP gene, so while under anhydrotetracycline (aTc) pressure, aTc will bind to TetR preventing the protein from binding to the aptamer, allowing translation to occur normally. Removal of aTc will result in a conditional knockdown of mtDNAP by blocking translation due to sequestration of the transcript mRNA.

Mechanism of Inducible Knockdown

The mechanism of the aptamer inducible knockdown system is a new and unique tool designed by Ganesan et al., providing controlled expression of the protein of interest (*45*). The plasmid is designed with a Tet repressor protein (TetR) and aptamers, which allow anhydrotetracycline (aTc)-dependent regulation of transcript. The aptamers are encoded within the 3'-untranslated regions (UTRs) of the target gene, so while under aTc pressure aTc will bind to TetR preventing the protein from binding to the aptamer, allowing translation to occur normally, shown in **Figure 3.3**. Without aTc, TetR will bind to the aptamer region of the messenger RNA transcript, blocking translation due to sequestration of the transcript mRNA resulting in a knockdown of the gene expression (**Figure 3.3**).

Their study demonstrated that by fusing TetR to DOZI (PF3D7_0320800), a putative ATP-dependent RNA helicase, there was increased translation regulation when used with a 3'-UTR aptamer. The TetR-DOZI system was successfully used to knockdown *P. falciparum* ATPase4, resulting in blood stage parasite death and establishing that the gene is essential (*45*).

Construction of the pMG75/mtDNAP plasmid resulted in loss of two aptamer copies, a common occurrence due to the repeating nature of the aptamer sequence, leaving eight copies in the final construct (data not shown). Studies by Ganesan et al. have demonstrated that a minimum of six copies is required for successful knockdown of the protein (personal communication), allowing the final pMG75/mtDNAP plasmid with eight copies to be used confidently in later experiments.

Generation of Transgenic Parasites

Transgenic parasites with integrated pMG75/mtDNAP plasmid DNA have aptamers encoded within the 3'-UTR of the mtDNAP gene. Integration of the pMG75/mtDNAP occurs by single crossover recombination with the parasite gDNA, utilizing the mtDNAP homology region within the plasmid. This mechanism relies on random double stranded breaks in the parasite gDNA for recombination to successfully occur, illustrated specifically in **Figure 3.4A**. As previously stated, the pMG75/mtDNAP plasmid is designed for single crossover

recombination to occur immediately before the stop codon of the mtDNAP gene within the 3'-UTR, allowing full replication and transcription of the integrated plasmid DNA to occur along with the parasite gDNA.

D10 parasites transfected with the pMG75/mtDNAP plasmid recovered after three weeks. Parasites were harvested for DNA, and PCR analysis was conducted to determine if integration of the plasmid was successful, as well as presence of resistant wild type parasites and length of the aptamer. The expected band sizes, illustrated in **Figure 3.4B**, are as follows: 1.3kb for integration, 1.8kb for resistant wild type parasites, and 2.4kb for full length aptamer with eight copies. Integration was not achieved immediately, therefore parasites were drug cycled for one full month to eliminate presence of episomal plasmid and select for parasites only with integrated plasmid DNA. After one drug cycle integration was successful, however only two copies of the aptamer remained, with PCR results shown in **Figure 3.4C**. Loss and deletion of aptamer copies has been previously observed by our laboratory, and tends be lost as stated due to the repetitive nature of the aptamer sequence.

In an attempt to generate parasites with full length aptamers, frozen stocks of parasites prior to the drug cycle were used for two more independent drug cycles. An additional transfection in D10 was conducted and drug cycled, as well as a transfection in NF54 with no drug cycling. DNA was collected from all parasite lines in various conditions, including independent transfections in either

D10 or NF54 wild type parasites, and if the parasites were drug cycled. PCR results using these parasite lines listed in **Table 3.1** are shown in **Figure 3.5**. The transfection in NF54 demonstrated integration with full aptamer, but was not used due to low integration signal compared to other parasite lines. Aptamer loss was observed again in the second D10 transfection with drug cycling, with no integration observed in the line prior to drug cycling. The repeated drug cycle of the first D10 transfection from frozen stocks provided a line with integration and full length aptamers. This line was used for cloning to eliminate resistant parasites with episomal plasmid from the culture. One transgenic clone, referred to as E6, was obtained with integration of the transgene bearing full length aptamers, and no wild type parasites, as determined by PCR reactions shown in **Figure 3.6**.

Transfections in D10 parasites were also conducted with the pUF-1/mtDNAP plasmid and three guides in the Cas2A plasmids. Integration of this plasmid occurs by double crossover recombination of the 3'- and 5'- homology regions after the cleavage of the gDNA by Cas9 determined by the specific guide RNAs. Parasites were recovered from transfection after seven weeks, but screening by PCR determined that only wild type parasites were present (data not shown).

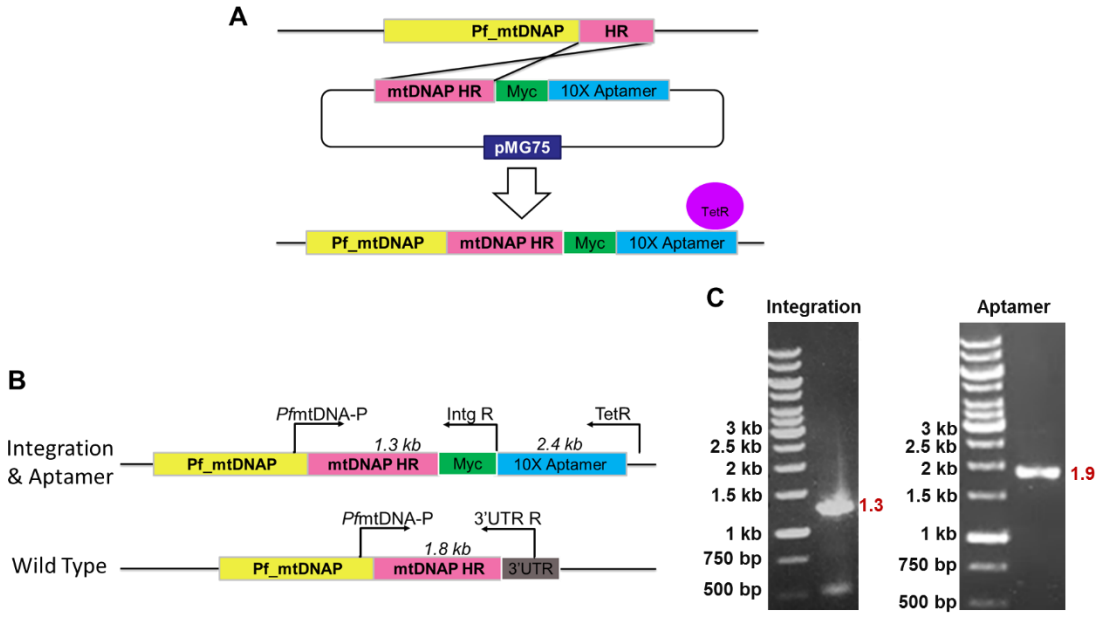

Figure 3.4: Generation of integrated transgenic parasites. **A.** Integration of the plasmid into the parasite genome by single crossover recombination with the 1kb homology region to the gDNA of the parasite, placing the plasmid within the 3'UTR after the mtDNAP gene. **B.** Primer binding regions and expected PCR band size for integration, aptamer length, and wild type, with specific primers listed in **Table 2.2**. **C.** D10 transgenic parasites after one drug cycle with integrated plasmid and two out of eight copies of the aptamer, confirmed by sequencing (data not shown).

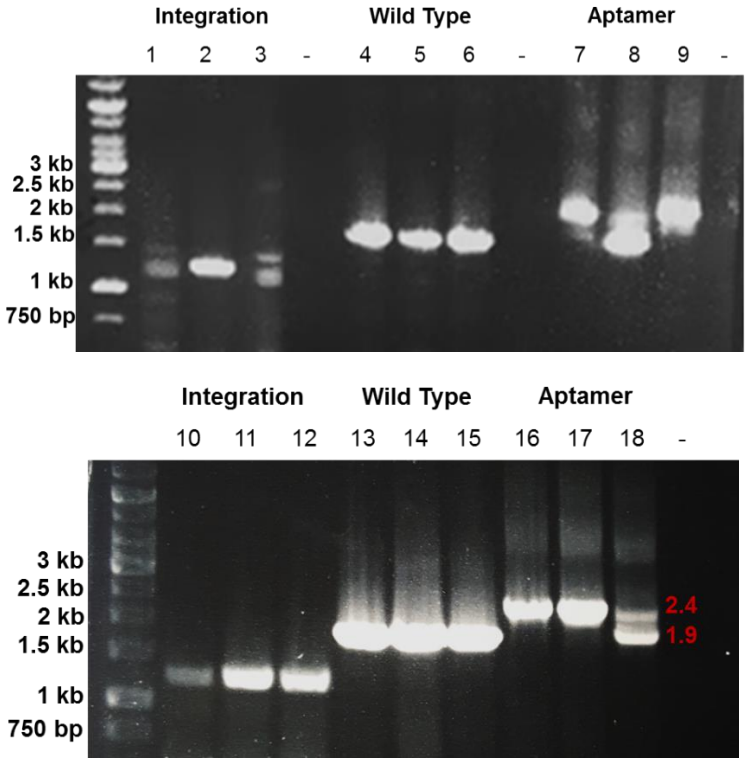

Figure 3.5: Generation of integrated transgenic parasites with full length aptamer. Multiple transfections and drug cycling completed to generate mixed culture parasites with integrated pMG75 plasmid DNA and eight copies of the aptamer as confirmed by PCR shown above, with primers listed above the lanes (**Table 2.2**) and lane contents indicated in **Table 3.1**. Expected band sizes are indicated in **Figure 3.4**.

Lanes	Transfection	WT Parasite Line	Integration?	Drug Cycle?	Aptamer Length
1, 4, 7	2	D10	N	N	-
2, 5, 8	1	D10	Y	Y	2
3, 6, 9	1	D10	N	N	-
10, 13, 16	3	NF54	Y	N	8
11, 14, 17*	**1**	**D10**	**Y**	**Y**	**8**
12, 15, 18	2	D10	Y	Y	2-4

Table 3.1: Generation of integrated transgenic parasites with full length aptamer. Characteristics of mixed culture transgenic parasite lines of multiple transfections, and some after one drug cycle, with PCR results demonstrated in **Figure 3.5**. Each row is indicative of an independent parasite culture. *Transgenic parasite line used for cloning.

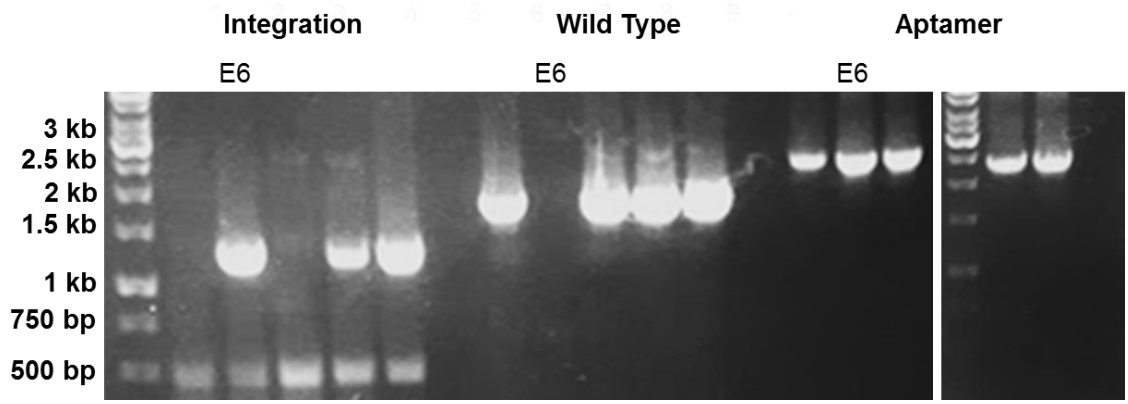

Figure 3.6: Screening of transgenic parasite clones. One successful clone was achieved, referred to as E6, indicated by presence of integrated plasmid and full length aptamer, and no presence of wild type parasites.

Localization of mtDNAP Could Not Be Determined

Immunofluorescent analysis (IFA) was used in an attempt to visualize the localization of the transgenic mtDNAP in the E6 clone. In multiple attempts of IFA staining of E6 parasites, the mtDNAP could not be visualized, whereas DAPI and mitochondrial staining was observed normally (data not shown). Successful protein tagging was demonstrated by Western blot analysis (**Figure 3.7**), however long exposure with the most sensitive substrate was required to visualize the protein, indicating very low expression of the mtDNAP. Due to this low level of expression and the availability of only a single Myc tag for IFA staining, it is likely that the protein cannot be visualized by IFA in the E6 clone. Protein tagging with

a triple HA tag might be a better option for IFA analysis due to increased binding sites and higher sensitivity, as discussed further in the next chapter.

mtDNAP Can Be Successfully Knocked Down

A knockdown assay was conducted with the E6 cloned parasites, with samples collected over the course of 20 days, or 10 cycles. Western blot analysis was conducted on samples from cycles 1, 4, 7 and 10, with and without aTc. The expected size of the mtDNAP is approximately 170 kDa. Protein expression at the proper size was observed in all samples in which aTc was maintained as shown in **Figure 3.7**. Knockdown was observed after the first cycle without aTc, and was maintained in the remaining cycles (**Figure 3.7**). Aldolase controls indicate presence of parasites in all samples, confirming appropriate loading of all samples. As previously mentioned, the protein expression of mtDNAP is relatively low, requiring a strong substrate and film exposure of at least five minutes in order to visualize Western blot bands. Due to this low expression, densitometry between the normal and knockdown Western blot bands cannot be conducted to determine the exact amount of knockdown that is occurring. Overall, knockdown of the mtDNAP was successful with no protein detectable by Western blots. Parasites were maintained over the course of 20 days with no parasite death observed, extending to over two months with no change in growth rate (data not shown). This observation suggests that the mtDNAP expression can be knocked

down below the detection level without affecting parasite survival in the asexual blood stage.

Knockdown of mtDNAP Does Not Alter Parasite Growth

Samples from the same knockdown assay described above were collected daily to determine the parasitemia over time. Parasitemia was determined by SYBR green staining of the parasite nuclear material and measurement with flow cytometry. The growth of the E6 parasites, with and without aTc, was plotted on a Log10 scale over time, shown in **Figure 3.8**. No difference in growth was observed between conditions, with a P-value of 0.9286 indicating no statistical significance (**Figure 3.8**). Knockdown was confirmed by Western blot (**Figure 3.7**), demonstrating that reducing the mtDNAP expression below the detection level does not affect the growth rate of the parasites.

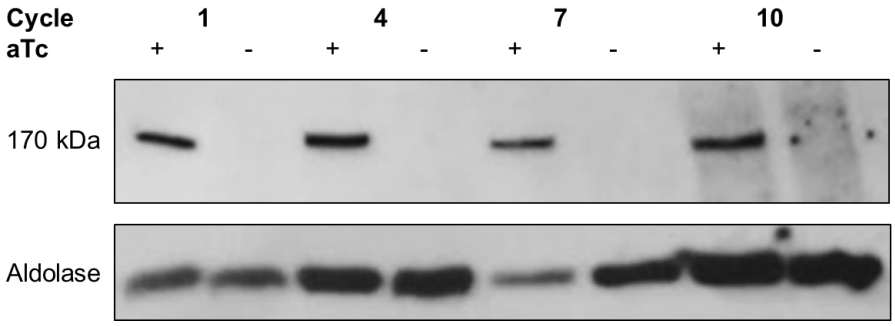

Figure 3.7: Western blot demonstrating knockdown of mtDNAP. Protein expression of mtDNAP can be observed in samples with aTc, with the expected size of 170 kDa, while samples without aTc have no observable protein expression. Aldolase is shown as a parasite loading control, observed in all samples.

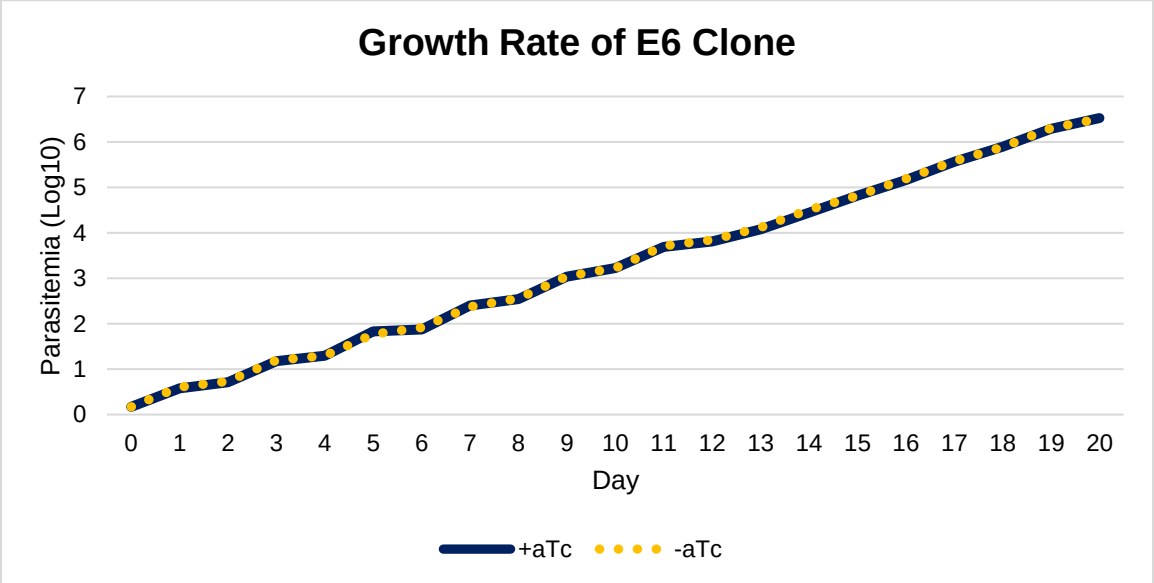

Figure 3.8: Growth rate of E6 clone is not affected by knockdown. No difference in growth was observed between parasites with and without aTc. Fixed parasite samples were stained with SYBR green to determine parasitemia by flow cytometry. The Log10 of the parasitemia was determined for each sample.

Transgenic Knockdown Parasites Demonstrate Decreased mtDNA

To further investigate the effect of mtDNAP knockdown, the amount of mtDNA present without the mtDNAP was determined using DNA samples collected during the knockdown assay. Knockdown of the mtDNAP is expected to result in less mtDNA, as there is no other annotated polymerase that could carry out mtDNA replication. The cytochrome *b* gene was used to quantify the mtDNA. The presence of cytochrome *b* was confirmed by PCR, with the gene being present in all samples from the knockdown assay (**Figure 3.9A**). Integration and length of aptamer were also confirmed by PCR, with integration and full aptamer maintained throughout the assay, eliminating the possibility of aptamer deletions affecting knockdown efficiency (**Figure 3.9B**). Quantitative PCR analysis was used to determine the copy number of the cytochrome *b* gene in parasites grown with or without aTc, using GAPDH as a nuclear gene control. The fold change in cytochrome *b* gene copy number was calculated for each cycle and controlled to parasites with aTc maintained.

The knockdown E6 parasites demonstrated decreased levels of cytochrome *b* copy number relative to normal E6 parasites with aTc, and therefore a decreased amount of mtDNA as expected (**Figure 3.10**). On average, there was approximately a 70% decrease in mtDNA observed in the E6 knockdown parasites. After the first cycle, knockdown parasites had 40% decrease in the mtDNA compared to parasites with aTc, with the fold change of mtDNA decreasing to

approximately 70% by the fourth cycle which was maintained in the remaining cycles. This confirms that knockdown of the mtDNAP does result in decreased mtDNA replication, demonstrated by the decreased amount of mtDNA. The remaining 30% of the mtDNA in knockdown parasites must be sufficient for parasite survival, and possibly indicates that the knockdown of mtDNAP is not complete, with some protein still being expressed.

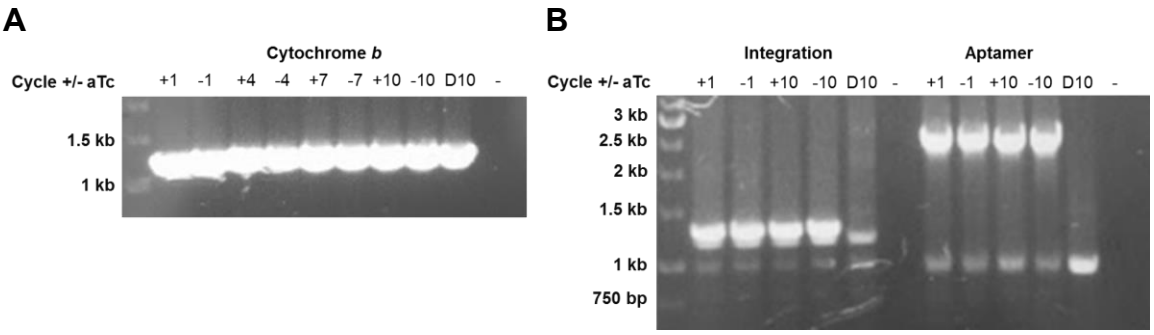

Figure 3.9: PCR analysis of knockdown assay. DNA samples were collected every cycle at trophozoite stage. PCR analysis of cytochrome *b*, integration and aptamer length were conducted. **A.** Cytochrome *b* is present in all samples. **B.** Integration and aptamer length were maintained throughout knockdown assay, as shown by cycle 1 and cycle 10 samples with and without aTc.

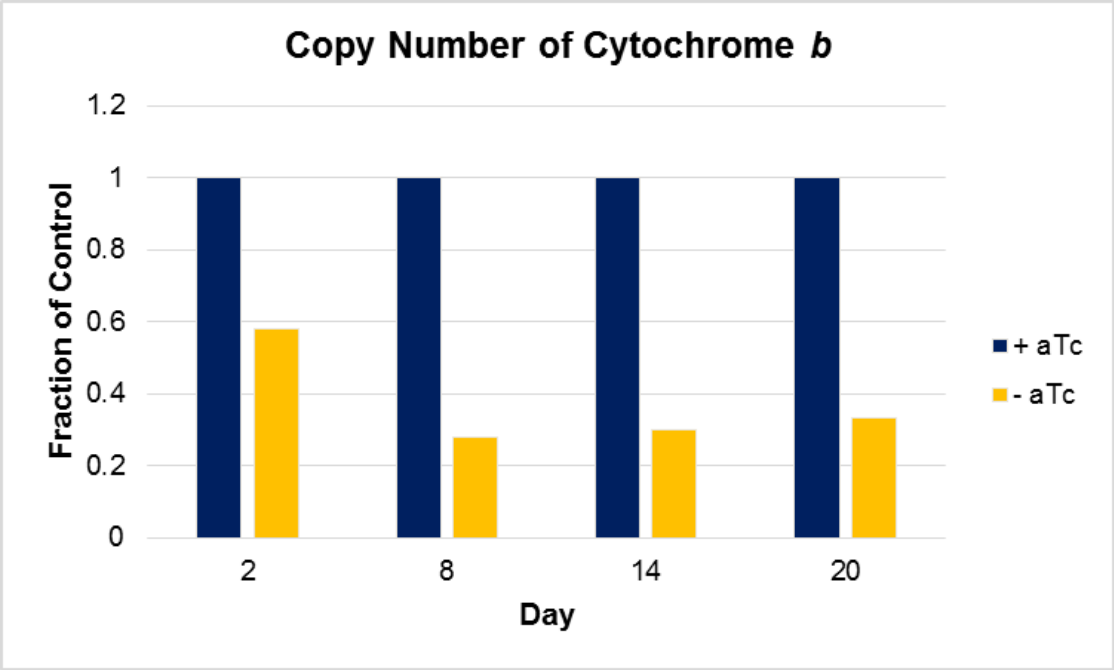

Figure 3.10: Quantitative PCR measurement of mtDNA copy number. DNA samples were collected from parasites grown with and without aTc from days 2, 8, 14, & 20 of the knockdown assay and used for qPCR analysis. Fold changes of the cytochrome *b* copy number of parasites without aTc compared to with aTc were plotted. Decreased copy number is observed in parasites without aTc.

Transgenic Knockdown Parasites Are Not Sensitive to Drug Inhibitors

The growth of transgenic E6 parasites and knockdown parasites was analyzed in the presence of atovaquone, proguanil, and ethidium bromide. A decreased amount of mtDNA should in theory decrease the protein expression of electron transport chain components encoded from the mtDNA, and thus can be expected to render the parasite more sensitive to atovaquone and proguanil. Ethidium bromide causes DNA damage, and was used to assess DNA repair activity of mtDNAP under normal and knockout conditions. The percentage of parasite growth was plotted against the concentration of each drug treatment (**Figure 3.11**). For all treatments, no difference was observed between normal and knockdown parasites, with death eventually resulting from all treatments at the indicated concentrations (**Figure 3.11**). This result further demonstrates that the remaining mtDNA in knockdown parasites is sufficient for survival, and does not alter the susceptibility of the parasites to drug inhibitors.

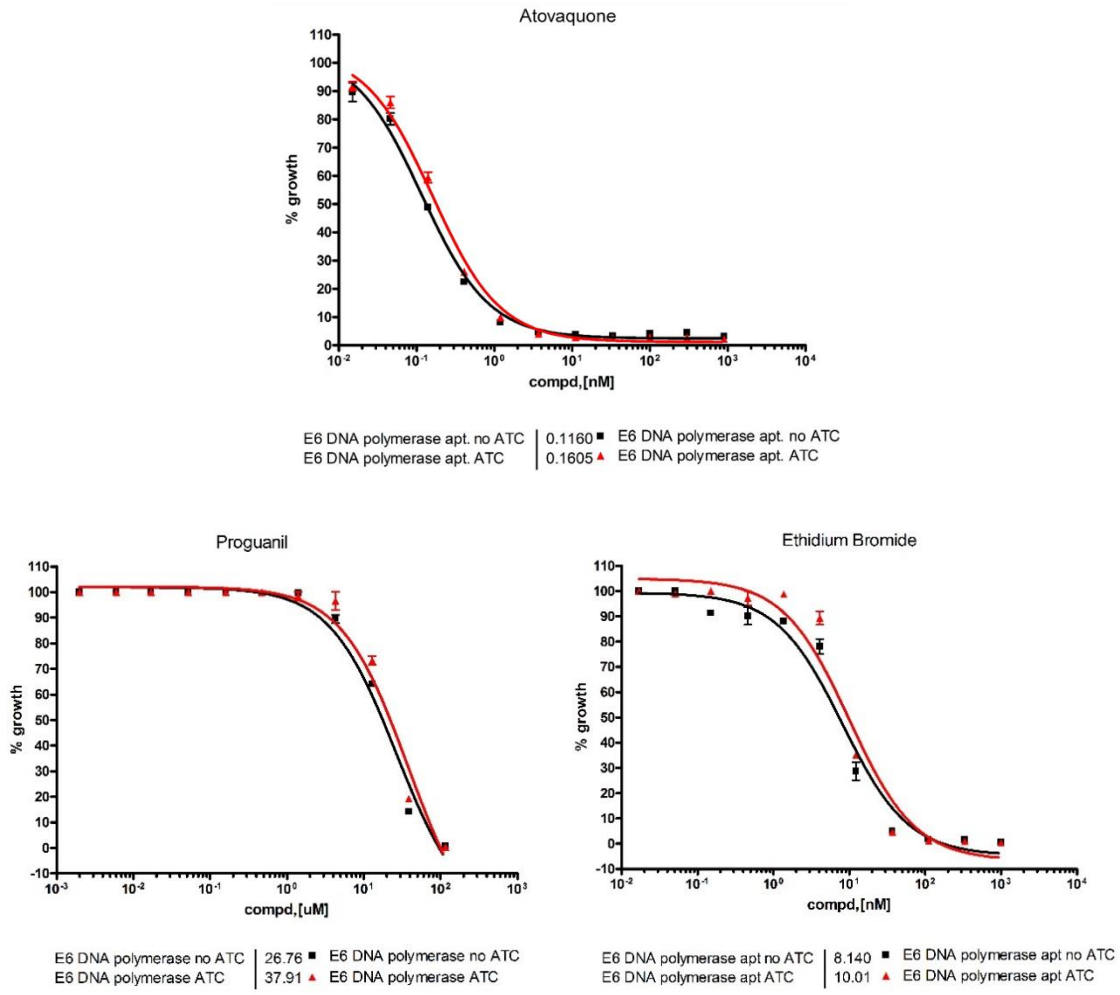

Figure 3.11: Growth inhibitory assays with various drug treatments. E6 parasites with and without aTc were treated independently with atovaquone, proguanil, and ethidium bromide. Percent growth is plotted against the amount of drug treatment, with IC_{50} values shown below the graph. No significant difference in IC_{50} was observed from any of the treatments between parasites with and without aTc.

Chapter 4: Discussion & Future Directions

Discussion

Limited research has been conducted on the mitochondrial DNA polymerase of *Plasmodium falciparum*, providing little insight into the nature of the protein, but allowing many possibilities for future studies. The aim of this thesis was to shed light on the mitochondrial DNA polymerase and characterize its functions through a genetic approach. The mtDNAP was successfully knocked down in *P. falciparum* blood stage parasites, demonstrating no growth phenotype after many cycles, as well as no change observed in response of the parasites to drugs targeting mitochondrial functions. However, the quantity of the mtDNA was decreased in knockdown parasites. Although the results were not as expected, it is consistent with the suggestion that this protein is in fact responsible for replicating the mitochondrial DNA, with the matter of its essentiality remaining left to be explored.

Generating a cloned line of transgenic parasites with the pMG75/mtDNAP plasmid proved difficult initially in the study, with aptamer deletions being the main issue. Aptamer deletions have been observed by our laboratory previously, and is likely due to the repetitive nature of the aptamer sequence. Given this knowledge, the aptamer length must be frequently checked to confirm its presence even after generation of cloned transgenic parasites. Eventually, a mixed culture with integration and a stable full length aptamer was able to be cloned, providing us with the E6 clone for experiments. During the knockdown assay using the E6

clone, lack of a growth phenotype was observed early on, as parasite death would be expected quickly should the mtDNAP be essential. Given this observation, the assay was carried over a full 10 cycles for adequate data collection.

Western blot analysis confirmed successful knockdown of the mtDNAP, with no protein expression detectable in any of the knockdown samples, compared to transgenic parasites with aTc maintained in culture. Given no signal in the knockdown parasites, it was initially assumed the knockdown was completely successful, and that the mtDNAP was non-essential for parasite survival. Upon mtDNA quantitation of samples from the assay, we discovered that the mtDNA was greatly reduced in knockdown parasites, indicating that the mtDNAP is in fact a polymerase that has a role in replicating the mtDNA. This led to many questions as to role of this protein and the actual level of knockdown that was achieved.

Typically, densitometry of Western blots is used to determine the exact level of knockdown achieved. In this case, that could not be conducted given that there was no observable protein expression at all by Western blot analysis. In order to observe protein expression in the transgenic parasites without knockdown, our most powerful substrate ("West Femto" reagent) plus long exposure times were required to see the faintest signal. This issue was also evident when conducting IFA analysis, as no observable signal was detected after multiple attempts of fluorescent staining of transgenic parasites. This leads us to believe that the mtDNAP is normally expressed at low levels, so in the case of a partial

knockdown, it is likely that an even lower level of expression would be undetectable. Given the presence of mtDNA in knockdown parasites, we suggest the knockdown to be partial, as a full knockdown would have resulted in no replication of the mtDNA, and thus no mtDNA remaining over time. Further study is required to determine the exact level of knockdown achieved, and alternative methods to generate a complete knockdown of the mtDNAP.

The mtDNA provides an essential role for *P. falciparum* blood stage parasites, encoding genes for the mtETC proteins cytochrome *c* oxidase subunits 1 and 3, and cytochrome *b* (*5, 30*). Without the mtDNA, parasites could not survive due to the reliance on the mtETC for pyrimidine biosynthesis and maintenance of the mitochondrial proton gradient (*29*). In the qPCR analysis of the knockdown mtDNA, there was on average a 70% decrease of the total mtDNA in knockdown parasites. With no change in growth rate observed, it is clear that approximately 30% of the mtDNA is sufficient for parasite health and survival. Parasites contain approximately 30 copies of the mtDNA, so it is possible that although the mtDNA is decreased, there are enough copies remaining for adequate protein production for the mtETC (*35*).

The knockdown parasites were unaffected by the various drug inhibitors. Atovaquone targets cytochrome *b* of the mtETC, a protein encoded by the mtDNA (*20*). Given the decreased amount of mtDNA in the knockdown parasites, overall protein expression could be decreased as well, resulting in the parasites with

greater susceptibility to the drug. We observed no change in growth in the presence of atovaquone, and also no difference with proguanil, a drug which disrupts the membrane potential generated by the mtETC (*30*). Although the mtDNA is decreased, there are multiple copies of the mtDNA normally present in the mitochondria, so the protein expression from the mtDNA in knockdown parasites is likely unaffected. Growth inhibitory assays were also conducted with ethidium bromide, a known DNA mutagen, to determine its effect on parasite growth and shed light on the possibility of the mtDNAP having DNA repair activity. Given there was no difference observed in knockdown parasites, it is likely that this assay is unlikely to assess mtDNA repair in parasites.

This thesis effectively characterized the putative mitochondrial DNA polymerase of *Plasmodium falciparum*. Ground work has been established to allow more in depth research is required to fully understand the role of this protein in parasite survival, a starting point has been established. We have successfully confirmed that the mitochondrial DNA polymerase does have a role in mitochondrial DNA replication, and hope to extend on these findings in future research.

Future Directions

The nature of this project was exploratory, and has provided many opportunities for future research. There were many issues with the low expression of mtDNAP. To alleviate this problem, a construct using a triple HA tag, rather than a single Myc tag, would be very beneficial for both western blot and IFA analysis. Utilizing a triple HA tag provides greater binding sites for antibodies, allowing better observation of the tagged protein. The pMG75 plasmid is currently being modified with the triple HA tag, for future use within our lab. This will provide greater insight into the localization of the mtDNAP, and more accurate western blot analysis of protein expression.

We believe that our knockdown was only partial, and a clean knockdown for future research is necessary. To achieve this, a double crossover recombination mechanism utilizing CRISPR-Cas9 to knock in the pMG75 plasmid will be utilized. This design allows the use of multiple guide RNAs, generating a line of transgenic parasites immediately without the requirement of cloning. This mechanism is less variable than the current single crossover mechanism, and should provide a line of transgenic parasites that can more effectively be knocked down with minimal variation.

Greater analysis of the mtDNA in knockdown parasites is crucial to fully understand the effect of decreased mtDNAP. The qPCR analysis will be repeated with increased biological replicates to determine the statistical significance of our

findings, as well as confirm results achieved in this study. Of interest is conducting sequencing of the mtDNA, to determine the exact copy number present in knockdown parasites and the possibility of mutations in the mtDNA as a result of the knockdown.

Lastly, we would like to knockdown the mtDNAP in the presence of yDHODH. As previously discussed, presence of yDHODH allows blood stage parasites to function independently of the mtETC (*30*). Due to the reliance of the mtDNA to produce essential proteins for the mtETC, we would expect the yDHODH to compensate for knockdown of the mtDNAP, allowing the production of a transgenic parasite line completely lacking the mtDNA. This has been a long term goal of the lab, and can be achieved utilizing the pMG75 mechanism in tandem with yDHODH background.

APPENDICES

>PF3D7_0625300 | Plasmodium falciparum 3D7 | DNA polymerase 1, putative | genomic | Pf3D7_06_v3 reverse | (geneStart+0 to geneEnd+1000) | length=5335

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ATAA

Appendix I. Binding regions of primers for the mtDNAP 1027 bp homology region cloned into the pMG75 vector. Primer binding regions are highlighted in yellow and the homology region highlighted in grey.

>PF3D7_0625300 | Plasmodium falciparum 3D7 | DNA polymerase 1, putative | genomic | Pf3D7_06_v3 reverse | (geneStart+0 to geneEnd+1000) | length=5335

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Appendix II. Binding regions of primers for the mtDNAP 5' and 3' homology regions cloned into the pUF-1 vector, as well as gRNA locations. Primer binding regions are as follows: 5'HR are highlighted in yellow, 3'HR highlighted in blue, flanking primers highlighted in green, and entire insert regions highlighted in grey. Guide RNAs are highlighted in purple.

CLUSTAL O(1.2.4) multiple sequence alignment

```
H. sapiens      ------------------------------------------------------------
P. falciparum   MKLFDSFFKHALIRINKRNIIYLNATRYYCNNINYNALINLLNKKNDINKEINALYSLLE

H. sapiens      ------------------------------------------------------------
P. falciparum   RLSNYKYKQYKDKLTLKNNINDEIKITNADKINNINIERDMNISHLDHHHNNHHHNNNHH

H. sapiens      ----------------------------------------------MSRLLWRKV-----
P. falciparum   NINHNNHHHNNHHNINHHNNHHNNHHNNNHFNDYKKLIDNWKNDKIKIFISWCPEIVEDK
                                                               : *

H. sapiens      ------------------AGATVGP----GPVPAPGRWVSSSVPASDP-----SDGQRRR
P. falciparum   YKSKCFSIPTYITFHIVITNNDIKLNNLLHNSHEYDDWNFNKIIQTINNQNNLKDKEKEK
                              :.  :            . *  ..:  :       .* ::.:

H. sapiens      QQQQQQQQQQ-----QQQPQQPQ------VL--SSEGGQLRHNPLDIQMLSRGLHEQIFG
P. falciparum   ENGQQHSQEYIGNCKKGESEIPSYDFKESLLEHINESSQLNHS-----ILSHKTKEQTHH
                :: **:.*:      : : : *.      :*   .*..**.*.     :**:  :** .

H. sapiens      QGGEMPGEAAVRRSVEHLQKHGLWGQ---------------------------------
P. falciparum   TNNNINGNYNN---DEHIEEEGKAKTKQNKTKNSIIEEKKKKTKKKKDEESHNDIINYTI
                 ..:: *:       **:::.*

H. sapiens      -------PAVPLPDVELRLPPLYGDNLDQHFRLLAQKQSLPYL--EAANLLLQAQLPPKP
P. falciparum   KKKTNTNNSLYNIESILNIPKTYEPNIHYDKCIHKEQNHIFFFSFNISELIINDQVKT--
                        ::   :  *.:*  *  *:. .  :  ::: : ::  : ::*::: *:

H. sapiens      PAWAWAEGWTRYGPEGEAVPVAIPEERALVFDVEVCLAEGTCPTLAVAISPSAWYSWCSQ
P. falciparum   ------------------------------KLNECIQQNFIKQNISNIHMDDLFLY---
                                           .:: *: :.        *  .  : :

H. sapiens      RLVEERYSWTSQLSPADLIPLE-----------VPT------GASSPTQRDWQEQLVVGH
P. falciparum   ----VVYDYKNLIHIFNNINLKLININNIFDIYIISSLIQLVQRGEKLQNVYNEYLNVKH
                      *.:.. :   : * *:           : :        ..  *. ::* * * *

H. sapiens      NVSFDRAH-------IREQYLIQGSRMRFLDTMSMHMAISGLSSFQRSLWIAAKQGKHKV
P. falciparum   KILIPNKINDIQNLSLHNFSYFSKFAPEFSDVISAKFGLYGWGKYQKKK-----DKKNKK
                :: : .         :::   :.    .* *.:* ::.: * ..:*:.      : *:*

H. sapiens      QPPTKQGQ--------------------------------------------------
P. falciparum   QTENHENNENYENNEYGKNNEYGKNNEYGKNNEYGKNNEYGKNNEYGKNNEYGKNNEYGK
                *  .::.:

H. sapiens      ---------------------------KSQRKARRGPAISSWDWLDISSVNSLAEVHRL
P. falciparum   NNVHNDDTYMDISNERKNKKSKEVKNKKKMEKKNKVEKEKQNYLSFTPHNINNLQDIKKL
                                          * ::* :     ..:  :   .:*.* ::::*

H. sapiens      YVGGPPLEKEPRELFVKGTMKDIRENFQDLMQYCAQDVWATHEVFQQQLPLFLERCPHPV
P. falciparum   VFGNKR-----------NISDITEE--DNICYSISRNC----CLILLFEYFINKLEHNI
                 .*.             .:.** *:  * : *. .        :   :  *:::  * :

H. sapiens      TLAGMLEMGVSYLPVNQNWERY--------LAEAQGTYEELQREMKKSLMD--LANDAC-
P. falciparum   NILN------LYIKVEQPLILCISHIEEKGIFLNQNKIEEIQKKSDDPLIYKNEIEELCK
                .: .       *: *:*             :   *.. **:*:: .. *:     :: *

H. sapiens      -QLLSGERYKEDPWLWDLEWDLQEFKQKKAKKVKKEPATASKLPIEGAGAPGDPMDQEDL
P. falciparum   CNINLNSSKQVSSLIYKQLLDI---------------------SISTD-HTEENMEDADE
                 ::  ..  : .  ::.   *:                      *.      : *:: *

H. sapiens      GPCSEEEEFQQDVMARACLQKLKGTTELLPKRPQHLPGHPGWYRKLCPRLDDPAWTPGPS
P. falciparum   HTDHQEEE-HVNDDNNECVDQLKAYTQTKEKERKDIYN----------------------
                    :*** : :   . *:::**. *:   *. :.: .
```

Appendix III. Protein sequence alignment of *H. sapiens* DNA polymerase subunit gamma-1 UniProtKB - P54098) and *P. falciparum* DNA polymerase 1 (PF3D7_0625300). Created by Clustal Omega multiple sequence alignment software.

LIST OF REFERENCES

1. L. S. Garcia, Malaria. *Clin Lab Med* **30**, 93-129 (2010).

2. G. W. H. Organization, *World Malaria Report 2016*. (2016).

3. S. B. Alassane Mbengue, Trupti Pandharkar, Haining Liu, Guillermina Estiu, Robert V. Stahelin, Shahir S. Rizk, Dieudonne L. Njimoh, Yana Ryan, Kesinee Chotivanich, Chea Nguon, Mehdi Ghorbal, Jose-Juan Lopez-Rubio, Michael Pfrender, Scott Emrich, Narla Mohandas, Arjen M. Dondorp, Olaf Wiest, Kasturi Haldar, A molecular mechanism of artemisinin resistance in Plasmodium falciparum malaria. *Nature* **520**, 683-687 (2015).

4. M. E. Milton, S. W. Nelson, Replication and Maintenance of the Plasmodium falciparum Apicoplast Genome. *Mol Biochem Parasitol*, (2016).

5. A. B. Vaidya, M. W. Mather, Mitochondrial evolution and functions in malaria parasites. *Annu Rev Microbiol* **63**, 249-267 (2009).

6. I. W. Sherman, *Malaria : parasite biology, pathogenesis, and protection*. (American Society for Microbiology, 1998), pp. 575.

7. N. J. White *et al.*, Malaria. *Lancet* **383**, 723-735 (2014).

8. D. A. Baker, Malaria gametocytogenesis. *Mol Biochem Parasitol* **172**, 57-65 (2010).

9. M. F. G. Louis. H Miller, Genevieve Milon, Malaria Pathogenesis. *Science* **264**, 1878-1883 (1994).

10. A. Bartoloni, L. Zammarchi, Clinical aspects of uncomplicated and severe malaria. *Mediterr J Hematol Infect Dis* **4**, e2012026 (2012).

11. M. F. Good, J. Currier, The importance of T cell homing and the spleen in reaching a balance between malaria immunity and immunopathology: the

moulding of immunity by early exposure to cross-reactive organisms. *Immunol Cell Biol* **70 (Pt 6)**, 405-410 (1992).

12. E. M. Riley, V. A. Stewart, Immune mechanisms in malaria: new insights in vaccine development. *Nat Med* **19**, 168-178 (2013).

13. D. J. Roberts *et al.*, Rapid switching to multiple antigenic and adhesive phenotypes in malaria. *Nature* **357**, 689-692 (1992).

14. E. A. Ashley *et al.*, Spread of artemisinin resistance in Plasmodium falciparum malaria. *N Engl J Med* **371**, 411-423 (2014).

15. N. J. White, Malaria: a molecular marker of artemisinin resistance. *Lancet* **383**, 1439-1440 (2014).

16. A. Mbengue *et al.*, A molecular mechanism of artemisinin resistance in Plasmodium falciparum malaria. *Nature* **520**, 683-687 (2015).

17. I. H. Cheeseman *et al.*, A major genome region underlying artemisinin resistance in malaria. *Science* **336**, 79-82 (2012).

18. S. Takala-Harrison *et al.*, Genetic loci associated with delayed clearance of Plasmodium falciparum following artemisinin treatment in Southeast Asia. *Proc Natl Acad Sci U S A* **110**, 240-245 (2013).

19. L. Roberts, Drug resistance triggers war to wipe out malaria in the Mekong region. *Science*. 2016 (10.1126/science.aaf9947).

20. I. K. Srivastava, A. B. Vaidya, A mechanism for the synergistic antimalarial action of atovaquone and proguanil. *Antimicrob Agents Chemother* **43**, 1334-1339 (1999).

21. A. B. Vaidya, M. W. Mather, Atovaquone resistance in malaria parasites. *Drug Resist Updat* **3**, 283-287 (2000).

22. N. Lane. pp. 1 online resource (xiii, 354 pages).

23. J. Krungkrai, The multiple roles of the mitochondrion of the malarial parasite. *Parasitology* **129**, 511-524 (2004).

24. D. Voet, J. G. Voet, *Biochemistry*. (ed. Fourth edition.), pp. xxv, 1,428, 453 pages.

25. H. Ke *et al.*, Genetic investigation of tricarboxylic acid metabolism during the Plasmodium falciparum life cycle. *Cell Rep* **11**, 164-174 (2015).

26. M. M. Moradian, D. Beglaryan, J. M. Skozylas, V. Kerikorian, Complete mitochondrial genome sequence of three Tetrahymena species reveals mutation hot spots and accelerated nonsynonymous substitutions in Ymf genes. *PLoS One* **2**, e650 (2007).

27. E. A. Nash, R. E. Nisbet, A. C. Barbrook, C. J. Howe, Dinoflagellates: a mitochondrial genome all at sea. *Trends Genet* **24**, 328-335 (2008).

28. A. B. Vaidya, R. Akella, K. Suplick, Sequences similar to genes for two mitochondrial proteins and portions of ribosomal RNA in tandemly arrayed 6-kilobase-pair DNA of a malarial parasite. *Mol Biochem Parasitol* **35**, 97-107 (1989).

29. A. B. Vaidya, M. W. Mather, A post-genomic view of the mitochondrion in malaria parasites. *Curr Top Microbiol Immunol* **295**, 233-250 (2005).

30. F. Seeber, J. Limenitakis, D. Soldati-Favre, Apicomplexan mitochondrial metabolism: a story of gains, losses and retentions. *Trends Parasitol* **24**, 468-478 (2008).

31. G. L. Nixon *et al.*, Targeting the mitochondrial electron transport chain of Plasmodium falciparum: new strategies towards the development of improved antimalarials for the elimination era. *Future Med Chem* **5**, 1573-1591 (2013).

32. M. Fry, M. Pudney, Site of action of the antimalarial hydroxynaphthoquinone, 2-[trans-4-(4'-chlorophenyl) cyclohexyl]-3-hydroxy-1,4-naphthoquinone (566C80). *Biochem Pharmacol* **43**, 1545-1553 (1992).

33. M. W. Mather *et al.*, Uncovering the molecular mode of action of the antimalarial drug atovaquone using a bacterial system. *J Biol Chem* **280**, 27458-27465 (2005).

34. W. E. Gutteridge, D. Dave, W. H. Richards, Conversion of dihydroorotate to orotate in parasitic protozoa. *Biochim Biophys Acta* **582**, 390-401 (1979).

35. H. J. Painter, J. M. Morrisey, M. W. Mather, A. B. Vaidya, Specific role of mitochondrial electron transport in blood-stage Plasmodium falciparum. *Nature* **446**, 88-91 (2007).

36. M. J. Gardner *et al.*, Mitochondrial DNA of the human malarial parasite Plasmodium falciparum. *Mol Biochem Parasitol* **31**, 11-17 (1988).

37. J. E. Feagin *et al.*, The fragmented mitochondrial ribosomal RNAs of Plasmodium falciparum. *PLoS One* **7**, e38320 (2012).

38. A. B. Vaidya, P. Arasu, Tandemly arranged gene clusters of malarial parasites that are highly conserved and transcribed. *Mol Biochem Parasitol* **22**, 249-257 (1987).

39. R. J. Wilson, D. H. Williamson, Extrachromosomal DNA in the Apicomplexa. *Microbiol Mol Biol Rev* **61**, 1-16 (1997).

40. A. H. Schinkel, H. F. Tabak, Mitochondrial RNA polymerase: dual role in transcription and replication. *Trends Genet* **5**, 149-154 (1989).

41. H. Ke, J. M. Morrisey, S. M. Ganesan, M. W. Mather, A. B. Vaidya, Mitochondrial RNA polymerase is an essential enzyme in erythrocytic stages of Plasmodium falciparum. *Mol Biochem Parasitol* **185**, 48-51 (2012).

42. P. Chavalitshewinkoon-Petmitr, S. Chawprom, L. Naesens, J. Balzarini, P. Wilairat, Partial purification and characterization of mitochondrial DNA polymerase from Plasmodium falciparum. *Parasitol Int* **49**, 279-288 (2000).

43. P. Chavalitshewinkoon *et al.*, Purification and characterization of DNA polymerases from Plasmodium falciparum. *Mol Biochem Parasitol* **61**, 243-253 (1993).

44. M. J. Gardner *et al.*, Genome sequence of the human malaria parasite Plasmodium falciparum. *Nature* **419**, 498-511 (2002).

45. S. M. Ganesan, A. Falla, S. J. Goldfless, A. S. Nasamu, J. C. Niles, Synthetic RNA-protein modules integrated with native translation mechanisms to control gene expression in malaria parasites. *Nat Commun* **7**, 10727 (2016).

46. C. J. Tonkin *et al.*, Localization of organellar proteins in Plasmodium falciparum using a novel set of transfection vectors and a new immunofluorescence fixation method. *Mol Biochem Parasitol* **137**, 13-21 (2004).

47. R. E. Desjardins, C. J. Canfield, J. D. Haynes, J. D. Chulay, Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. *Antimicrob Agents Chemother* **16**, 710-718 (1979).

48. J. Filee, P. Forterre, T. Sen-Lin, J. Laurent, Evolution of DNA polymerase families: evidences for multiple gene exchange between cellular and viral proteins. *J Mol Evol* **54**, 763-773 (2002).

49. A. Marchler-Bauer *et al.*, CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Res* **45**, D200-D203 (2017).